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(57) Abstract

This invention relates to vaccines and diagnostics and more particularly to vaccines and diagnostics which employ proteins and/or fragments and/or derivatives thereof having homology to heat shock proteins of Trypanosoma cruzi.

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VACCINE DIAGNOSTIC EMPLOYING PROTEINS HOMOLOGOUS TO HEAT SHOCK PROTEINS OF TRYPANOSOMA CRUZI

BACKGROUND OF THE INVENTION

This invention relates to vaccines and diagnostics and more particularly to vaccines and diagnostics which employ proteins and/or fragments and/or derivatives thereof having homology to heat shock proteins of Trypanosoma cruzi.

Heat shock proteins, sometimes referred to as stress proteins, have been found in a wide variety of cells, and have been generally described in an article written by Tissieres on pages 419 through 429 of "Heat Shock from Bacteria to Man" (Cold Spring Harbour Laboratory, 1982).

DESCRIPTION OF THE FIGURES

Figure 1 provides the gene and derived amino acid sequence for the Hsp70 antigen of T. cruzi.

Figure 2 provides an alignment of heat shock proteins from a variety of organisms: 1. M. hyopneumoniae, 2. Bacillus megaterium, 3. Escherichia coli, 4. T. cruzi, 5. T. cruzi, 6. Rat, 7. Xenopus laevis 8. human, 9. chicken, 10. Zea mays, 11. Serratia marcescens.

Figure 3 provides a restriction map of pMYC016 containing the full length gene for the Hsp70 antigen of M. hyopneumoniae.

Figure 4 provides an intermediate plasmid for the expression of the Hsp70 antigen of M. hyopneumoniae.

Figure 5 provides the gene and derived amino acid sequence for the Hsp70 antigen of M. hyopneumoniae.

Figure 6 provides restriction map of pMYC029 which is a low level expression plasmid containing the

full length gene for the Hsp70 antigen of M. hyopneumoniae.

Figure 7 provides a restriction map of pMYC031 which is a high level expression plasmid containing the full length gene for the Hsp70 antigen of M. hyopneumoniae.

Figure 8 provides a restriction map of pCAM101 containing the trpT176 gene.

Figure 9 provides a restriction map of pMYC032 which is an expression plasmid containing the full length gene for the Hsp70 antigen of M. hyopneumoniae and the trpT176 gene.

Figure 10 provides a restriction map of pMGA4 which is an expression plasmid containing the full length gene for the Hsp70 antigen of M. gallisepticum.

Figure 11 provides the gene and derived amino acid sequence for the Hsp70 antigen of M. hyopneumoniae.

Figure 12 provides a restriction map of pMGA10 which is an expression plasmid containing the full length gene for the Hsp70 antigen of M. hyopneumoniae and the trpT176 gene.

SUMMARY OF THE INVENTION

Vaccines are disclosed for the protection against organisms which comprise a physiologically acceptable carrier with a protein which is capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism, the native protein having at least 50% hpmology with a heat shock protein of T. cruzi. Processes for protecting a host against an organism are also disclosed which comprise administering an effective amount of a protein capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism, the native protein

having at least 50% homology with a T. cruzi heat shock protein.

Further disclosed are processes for determining an organism in a host which comprise contacting a sample derived from a host containing an organism or suspected of containing an organism with an antibody or antibody fragment which recognizes at least one epitope of a native protein present in the organism, the native protein having at least 50% homology with a heat shock protein of T. cruzi; and determining protein present in the organism bound to the antibody.

For such vaccines and processes, the native protein referred to above may be derived from a species of Mycoplasma, Mycobacteria or Trypanosoma, provided that the native protein is not derived from Trypanosoma cruzi. Preferably, the native protein of Mycoplasma derivation is one selected from the group consisting of M. mycoides, M. bovis, M. bovigenitalium, M. bovoculi, M. bovirhinis, M. dispar, M. hyorhinis, M. hyosynoviae, M. hyopneumoniae, M. gallisepticum, M. pneumoniae, and M. synoviae, most preferably from M. hyopneumoniae and M. gallisepticum. The native protein of Mycobacteria derivation is preferably one selected from the group consisting of M. bovis, M. leprae, and M. tuberculosis.

The recombinant sequence of nucleic acid encoding the heat shock proteins of M. hyopneumoniae and M. gallisepticum is also disclosed.

DETAILED DESCRIPTION

Applicant has found that certain heat shock proteins and/or fragments and/or derivatives thereof may be employed in a vaccine to protect against an organism containing such heat shock protein.

Applicant has further found that certain heat shock proteins and/or fragments or derivatives thereof, as well as antibodies produced in response

to such heat shock proteins and/or fragments or derivatives thereof may be employed as a diagnostic for determining an organism containing such heat shock proteins.

Applicant has also found that certain DNA (RNA) sequences encoding for a heat shock protein of an organism may be employed as a diagnostic for determining the organism.

In accordance with the one aspect of the present invention, there is provided a vaccine for protecting against an organism which includes a heat shock protein wherein the vaccine includes a protein capable of eliciting an antibody which recognizes at least one epitope of a heat shock protein of the organism which heat shock protein of the organism has at least 50% homology with a heat shock protein of Trypanosoma cruzi (T. cruzi).

In accordance with another aspect of the present invention, there is provided a process for protecting against a disease caused by an organism which includes a heat shock protein by administering to a host at least one protein capable of eliciting an antibody which recognizes at least one epitope of a heat shock protein of the organism which heat shock protein of the organism has at least 50% homology with a heat shock protein of Trypanosoma cruzi (T. cruzi).

The term that an antigen or protein has at least 50% homology with a heat shock protein of T. cruzi, as used herein, means that on a position by position basis, at least 50% of the amino acids of the heat shock protein of T. cruzi are also present in the antigen or protein.

More particularly, in a preferred embodiment the heat shock protein or polypeptide of T. cruzi with which an antigen or protein is to have at least 50% homology is at least one of the T. cruzi heat shock

proteins having a molecular weight of about 70 kD, or about 85 kD or about 65 kD, preferably the heat shock protein having a molecular weight of about 70 kD.

The T. cruzi heat shock protein having a molecular weight of about 70 kD may be prepared as described in Example 1. The amino acid and DNA sequence for the 70 kD protein is shown in Figure 1 of the drawings, with the 70 kD protein starting at base pair 25 and terminating at base pair 677.

The T. cruzi heat shock protein having a molecular weight of about 85 kD is described by Dragon et al. Molecular and Cellular Biology, Volume 7 No. 3 Pages 1271-75 (March 1987).

The protein which is present in the organism and which is at least 50% homologous to a T. cruzi heat shock protein will sometimes be referred to herein as the "homologous protein" or the "homologous heat shock protein".

The protein employed in formulating the vaccine for protection against an organism may be identical to a homologous protein present in the organism to be protected against, or may be a fragment or derivative of such homologous protein, provided that the protein which is used in the vaccine is capable of eliciting an antibody which recognizes at least one epitope of the homologous protein. For example, the protein employed in the vaccine may be only a portion of the homologous protein present in the organism or may have one or more amino acids which differ from the amino acids of the homologous protein in the organism or may be the homologous protein (or fragment or derivative thereof) fused to another protein.

The term "protein which is capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism, said native protein having at least 50% homology with a heat shock protein of T. cruzi" (such protein present

in the organism is what is sometimes referred to as the homologous protein) encompasses the homologous protein present in the organism or a fragment of such homologous protein or a derivative of such homologous protein or a fusion product of such homologous protein (or fragment or derivative thereof) with another protein. As should be apparent, the protein or proteins included in the vaccine may include more or less amino acids or amino acids different from the amino acids of the homologous protein present in the organism.

The protein or proteins employed in the vaccine may be identified and produced by recombinant techniques. More particularly, the DNA (or RNA) encoding for a T. cruzi heat shock protein is employed as a probe to identify DNA present in the organism against which protection is sought which has at least 50% homology with the DNA (RNA) encoding for a T. cruzi heat shock protein. The DNA of the organism having the requisite homology is sometimes referred to herein as the "homologous DNA".

The homologous DNA of the organism identified by such probe is employed to produce homologous protein of the organism by recombinant techniques. Thus, for example, the DNA encoding for the protein of Figure 1 may be suitably labeled, for example with ^{32}P, by procedures known in the art to thereby provide a probe for identifying DNA in the organism having at least 50% homology with the DNA sequence encoding for the protein of Figure 1.

Figure 2 presents an alignment of the amino acid sequences of Hsp70 proteins from a number of species. The amino acids are depicted by their single letter abbreviations. Stretches of sequence identical in all examined species were identified (denoted by upper case text in the consensus sequence depicted below the individual sequences). Several regions

containing sequences at least six amino acids in length which were identical in all Hsp70 sequences. For example, between amino acid 138 and 209 of T. cruzi lie the sequences TVPAYF, RIINEPTA, and DLGGGTFD which are conserved in Hsp70 sequences. The DNA sequences which could encode these conserved sequences were determined. The 17-mer nucleotide sequences having low coding degeneracy serve as universal oligonucleotide probes for Hsp70 genes.

The probing conditions selected are such that hybrids are identified in which there is at least 50% homology between the selected DNA probe which encodes for a T. cruzi heat shock protein and the DNA being probed for in the organism. Such probing is done at relatively low stringency. Low stringency is achieved by known methods such as reduced temperature and increased salt concentrations (e.g., hybridizing at 37°C and 5-6 X standard salt-citrate buffer or 5-6X standard salt-EDTA-Tris buffer).

The selected homologous DNA of the organism may be included in any of a wide variety of vectors or plasmids for producing a protein to be employed in formulating a vaccine against the organism. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences; e.g., derivatives of SV40; bacterial plasmids; phage DNA's; yeast plasmids; vectors derived from combinations of plasmids and phage DNAs, viral DNA such as vaccinia, adenovirus, fowl pox, virus, pseudorabies, etc.

The appropriate DNA sequences may be inserted into the vector by a variety of procedures. In general, the DNA sequences are inserted into an appropriate restriction endonuclease site by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequences in the vector are operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli lac or trp, the phage lambda PL promoter and other promoters known to control expression of genes in prokaryotic and eukaryotic cells or their viruses.

The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequences as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Salmonella typhimurium, fungal cells, such as yeast; animal cells such as CHO or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

The expression vehicle including the appropriate DNA sequences for the protein to be expressed and the t-RNA inserted at the selected site may include a DNA or gene sequence which is not part of the gene coding for the protein. For example, the desired DNA sequence may be fused in the same reading

frame to a DNA sequence which aids in expression or improves purification or permits increases in the immunonogenicity.

In employing recombinant techniques for producing the active protein, purifications, digestions, ligations and transformations may be accomplished as described in "Molecular Cloning: A Laboratory Manual" by Maniatis et al., Cole Spring Laboratory, 1982 ("Maniatis"). In addition, transformations may be accomplished by the procedure of Cohen, PNAS, 69:2110 (1973).

When seeking to develop a vaccine, neutralizing or protective antibodies could be targeted toward discontinuous, conformation-dependent epitopes of the native antigen. One must therefore consider whether the protein obtained from the recombinant expression system might have a three dimensional structure (conformation) which differs substantially from that of the original protein molecule in its natural environment. Thus, depending on the immunogenic properties of the isolated proteins, one might need to renature it to restore the appropriate molecular conformation. Numerous methods for renaturation of proteins can be found in the scientific literature and include; 1) denaturation (unfolding) of improperly folded proteins using agents such as alkali, chaotropic agent, organic solvents, and ionic detergents followed by a renaturation step achieved by dilution, dialysis, or pH adjustment to remove the denaturant, and 2) reconstitution of proteins into a lipid bilayer or liposome to re-create a membrane like environment for the immunogenic protein.

The vaccine which includes a protein of the type hereinabove described may be employed in a vaccine for protecting against diseases caused by a wide variety of organisms. Table 1 provides representative examples of such organisms. Of particular interest are

species of Trypanosoma, Mycoplasma and Mycobacteria. Trypanosoma and Mycoplasma heat shock proteins are described herein. Heat shock proteins for Mycobacteria are known. Young et al., P.N.A.S. (USA), 85:4267-4270 (1988).

A host may be protected against a disease caused by a certain organism by incorporating into the vaccine a protein which is capable of eliciting antibodies which are recognized by at least one epitope of a homologous protein of the organism. As hereinabove indicated the protein which is capable of eliciting such antibodies (hereinafter sometimes referred to as the active protein) may correspond to the homologous protein of the organism or may be a fragment or derivative thereof. As should be apparent, if the disease against which a host is to be protected is Chagas, which is caused by T.cruzi, the protein which is included in the vaccine would be one or more heat shock proteins of T. cruzi or a fragment or derivative thereof capable of eliciting antibodies which recognize an epitope of T. cruzi heat shock protein. The host which is protected is dependent upon the organism against which protection is sought. In general, the host is an animal (either a human or nonhuman animal) which is subject to a disease caused by the organism. Thus, for example if the organism against which protection is sought is one which is known to cause disease in man, then the vaccine including the active protein or proteins would be administered to a human host. If the organism is known to cause a disease in a nonhuman animal, then the vaccine including the active protein would be administered to a nonhuman animal.

In formulating a vaccine, the active protein is employed in the vaccine in an amount effective to provide protection against the disease caused by the organism against which protection is sought. In

general, each dose of the vaccine contains at least 5 micrograms and preferably at least 100 micrograms of the active protein. In most cases, the vaccine does not include the active protein in an amount greater than 20 milligrams.

The term "protection" or "protecting" when used with respect to a vaccine means that the vaccine prevents the disease or reduces the severity of the disease.

The active protein is employed in conjunction with a physiologically acceptable vehicle to provide protection against the organism. As representative examples of suitable vaccines in carriers, there may be mentioned: mineral oil, alum, synthetic polymers, etc. Vehicles for vaccines are well known in the art and the selection of a suitable vehicle is deemed to be within the scope of those skilled in the art from the teachings herein. The selection of a suitable vehicle is also dependent upon the manner in which the vaccine is to be administered. The vaccine may be in the form of an injectable dose and may be administered intra-muscularly, intravenously, or by sub-cutaneous administration. It is also possible to administer the vaccine orally by mixing the active components with feed or water; providing a tablet form, etc.

Other means for administering the vaccine should be apparent to those skilled in the art from the teachings herein; accordingly, the scope of the invention is not limited to a particular delivery form.

It is to be understood that a vaccine may also be formulated by use of an antibody elicited in response to a homologous protein of the organism.

The protein and/or antibody used in the vaccine is essentially free of the organism; i.e., cellular matter.

In accordance with another aspect of the present invention, there is provided a diagnostic kit and/or assay for determining an organism which employs in the assay and/or kit an antigen which is recognized by an antibody elicited by a protein of the organism which has at least 50% homology with a T. cruzi heat shock protein, as hereinabove described, i.e., a "homologous protein" of the organism.

The antigen employed as a diagnostic may be obtained or produced as hereinabove described with reference to the active protein included in the vaccine.

In accordance with yet a further aspect of the present invention, there is provided a diagnostic assay and/or reagent for determining an organism which includes and/or employs an antibody (or fragment thereof) which recognizes an antigen of the organism to be determined, which antigen of the organism has at least 50% homology with a heat shock protein of T. cruzi, as hereinabove described.

The antibody employed in the assay and/or assay kit may be either a polyclonal or monoclonal antibody elicited in response to a homologous protein. In particular, the antibody employed in the diagnostic assay and/or kit is elicited in response to a protein and/or fragment and/or derivative thereof having at least 50% homology with a heat shock protein of T. cruzi.

A diagnostic kit and/or assay for determining an organism which includes a homologous protein may be formulated to determine such organism by a variety of procedure.

For example, the organism may be determined by a so-called sandwich assay kit or assay for determining the organism by determining in a sample (derived from a host containing or suspected of

containing the organism) antibody elicited in response to a homologous protein of the organism. In this procedure, antigen of the type hereinabove described is contacted with the sample under conditions at which any of such antibody present in the sample is immunobound to the antigen, which antigen is preferably supported on a solid support.

Antibody bound to such antigen may then be determined by use of an appropriate tracer comprised of a ligand bound or recognized by such antibody labeled with a detectable marker or label. The ligand of the tracer may be, for example, an antibody bound by or recognized by the bound antibody.

The marker may be any one of a wide variety of labels (for example a radioactive label, an enzyme label, a chromogen label, etc.).

The techniques for forming such an assay and for providing a tracer are known in the art and no further details in this respect are deemed necessary for understanding the present invention.

For example, there may be employed a so-called ELISA sandwich assay format in which a plastic microtiter plate is coated with an antigen of the type described (one which is recognized by antibody elicited in response to homologous protein of the organism) and sample derived from a host suspected of containing the organism is incubated with the coated antigen. After appropriate washing, labeled immunoglobulin (antiglobulin to the host species which is suspected of containing the organism) labeled with a detectable enzyme (for example horseradish peroxidase or alkaline phosphatase) is incubated with the antibody bound by the coated antigen. After washing, an appropriate developer is added.

Alternatively, an agglutination assay may be employed in which case particles, such as polystyrene

beads, coated with the appropriate antigen is mixed with appropriate sample, and presence of antibody is detected by agglutination.

These and other procedures should be apparent to those skilled in the art.

In an alternative sandwich immunoassay format, an antibody of the type hereinabove described may be employed to directly determine a homologous heat shock antigen or protein of the organism to be determined.. For example, a sample (derived from a host containing or suspected of containing the organism) is subjected to a sandwich assay by contacting the sample with an antibody (or fragment thereof) which recognizes the homologous heat shock antigen of the organism, which antibody is preferably supported on a solid support. Such contacting is effected under conditions which will immunobind the homologous heat shock antigen (if present) to the antibody. Thereafter, bound antigen may be determined by use of a tracer comprised of a ligand (which is bound by or recognizes the homologous antigen) labeled with a detectable marker or label. Thus, for example, the tracer may be labeled antibody elicited in response to the homologous antigen of the organism. As hereinabove indicated, the antibodies capable of recognizing a homologous protein of the organism may be a monoclonal and/or polyclonal antibody.

In this assay format, which employs an antibody which recognizes a homologous protein of the organism, markers (labels) and techniques, as hereinabove described and as known in the art, may also be employed.

The assay or reagent kit which employs antigen and/or antibody of the type hereinabove described may be included in an appropriate reagent kit package. The package may include other materials

useful in the assay, for example, tracer, buffers, standards, etc., in appropriate reagent containers.

In accordance with another aspect of the present invention, there is provided an assay and/or reagent kit for determining the presence of an organism which includes or employs a DNA probe which encodes for a protein of the organism having at least 50% homology with a heat shock protein of T. cruzi as hereinabove described.

The DNA probe which is used may be all or a portion of the DNA which encodes for a homologous protein. If a portion of the DNA which encodes for a homologous protein is employed, such DNA portion should include a portion of the DNA which encodes for a variable region of the homologous protein.

Accordingly, the DNA probe is employed under conditions whereby hybridization is accomplished over at least a portion of the DNA which encodes for a variable region (preferably a hypervariable region) of the homologous protein.

The hydridization may be performed with a suitably labeled form of the DNA (for example ^{32}P, although other detectable labels, including non-radioactive labels may be used) in a procedure similar to the procedure for identifying DNA of the organism encoding for a protein having the requisite homology with a T. cruzi heat shock protein.

The present invention will be further described with respect to the following examples; however, the scope of the invention is not to be limited thereby. Unless otherwise indicated, all methods and abbreviations are well known in the art and are found in Maniatis. All references in this document are hereby incorporated by reference herein.

Example 1 -- Trypanosoma Cruzi Heat Shock Protein and Its Reaction with Sera from Infected Persons.

A. Growth and Isolation of Parasites

Trypanosoma cruzi, Peru strain, was used in all experiments. Epimastigotes were grown at 28°C in modified HM (Warren, S. Parasitology, 46:529-539, 1960); 37 g/1 brain heart infusion (Difco Lab., Detroit, MI), 2.5 mg/1 hemin, 10% heat-inactivated fetal calf serum. Log phase cells were harvested by centrifugation and washed twice with cold PSG (20 mM sodium phosphate, pH 7.4, 0.9% NaCl, 1.0% glucose). Culture form trypomastigotes were obtained from infected Va-13 cells as previously described. See Sanderson et al., Parasitology, 80:153-162, (1980), and Lanar and Manning, Mol. and Biochem., Parasitology, 11:119-131, (1984).

B. Isolation of DNA and RNA

Parasites were harvested from culture by centrifugation and washed several times with PSG (20 mM sodium phosphate, pH 7.4, 0.9% NaCl, 1.0% glucose). Epimastigotes were resuspended at a concentration of 10^9/ml in PEG/EGTA buffer (20 mM Tris-HCl, pH 7.6, 25 mM EGTA, 50 mM MgCl, 25mM CaCl, 1.0% Triton-X100, and 4mM dithiothreitol), plus 250 u/ml of RNAS in (Promega Biotec, Madison, WI), incubated on ice for 20 min., centrifuged at 8000 x g for 15 minutes at 4°C. The supernatant containing the RNA was phenol extracted 3 times, then extracted once with chloroformisoamyl alcohol (24:1) and ethanol precipitated. The pellet (nuclei and kinetoplasts) was resuspended at a concentration of 10^9 parasite equivalents/ml in 10 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.1% SDS, 150 ug/ml Proteinase K (Boehringer- Mannheim, Indianapolis, IN) and incubated at 65°C for 1 hour. After cooling to room temperature, the DNA was gently extracted with an equal volume of phenol for 1 hour. This extraction

was repeated once, and the aqueous phase was extracted with chloroform-isoamyl alcohol (24:1) once. The DNA was recovered by ethanol precipitation. The DNA pellet was gently redissolved in 10 mM Tris-HCl. pH 8.0. 1 mM EDTA and treated with 0.15 mg/ml DNAse-free RNAse A for 30 minutes at room temperature. After RNAse digestion the sample was extracted once with phenol, once with chloroformisoamyl alcohol, and then precipated with ethanol. The size of the DNA was determined to be greater than 20 kilobase pairs (kb) on agarose gels. Trypomastigote DNA and RNA was prepared in an identical manner except that the parasites were resuspended at a concentration of 5 x 10^9/ml.

C. Preparation of A+ mRNA

Poly A+ containing RNA was isolated by Oligo(dT)-cellulose chromatography (Aviv and Leder, J. Immunol., 127:855-859, 1972). Total RNA was loaded onto an oligo (dT)-cellulose column (Type 3, Collaborative Research, Lexington, MA) in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% SDS, 400 mM LiCl. RNA was eluted from the column at 40°C with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% SDS.

D. Construction of the T. cruzi "Sau3a Partial" Genomic Library in Bacteriophage EMBL3

200 µg of T.cruzi epimastigote DNA was digested with the restriction endonuclease Sau3A (Boehringer-Mannheim, Indianapolis, IN) according to manufacturer's specifications. Aliquots of the reaction were removed at 1, 2.5, 5, 10, 20, 40 and 60 minutes. Upon removal each aliquot was diluted to 25 mM in EDTA and heated for 15 minutes at 68°C. The samples were pooled, the DNA was size fractionated over a Sephacryl S-1000 column (Pharmacia, Piscataway, NJ) in 200 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA. Those fractions containing DNA in size from 5 kb to 20

kd were pooled, ethanol precipiated, and used for cloning. The lambda bacteriophage cloning vector EMBL3 (Frishauf et al., J. Mol. Biol., 170:827-842, 1983) was used. EMBL3 arms and GIGAPAK packaging system were purchased from Vector Cloning Systems (San Diego, CA) and used according to the manufacturer's instructions.

E. Hybridization-Selection/Translation

Specific T. cruzi RNAs were purified from total T. cruzi RNA using the technique of hybridization-selection/translation as described by Riccardi et al., PNAS, 76:4927-4931, 1972. 25-50 ug of purified plasmid DNA was digested with an appropriate restriction endonuclease (to linearize the plasmid), the DNA was cleaned by phenol extraction and chloroform extraction and denatured by boiling for 10 minutes. Following boiling, the DNA was quick-frozen, thawed, then spotted onto a 9mm diameter nitrocellulose filter. The filter was washed several times with 6XSSC, then air dried and baked for 2 hours at 80°C in vacuo. For hybridization, 100 μg of T. cruzi total RNA was reacted with the DNA containing filter in a solution containing 65% formamide, 0.01 M PIPES, pH 6.4, 0.4 M NaCl at 65°C for 3 hours. Following the hybridization reaction, the filter was washed 10 times with 1XSSC, 0.1% SDS at 60°C, 3 times with 0.002 M EDTA at 60°C, and once with water at room temperature. The specifically hybridized mRNA is eluted from the filter by boiling the filter in a small volume of water for two minutes, quick-freezing the solution, then ethanol precipitating the RNA. The purified RNA is resuspended in water, then translated in an in vitro translation system (such as rabbit reticulocyte).

F. Immunoprecipitation Reactions

A 1:10 to 1:50 dilution of individual serum was prepared using the 10 mM Tris-HCl, pH7.5, 1% Nonidet

P-40 (NP 40), 1 mM N-alpha-p-tosyl-L-Lysine chloromethyl ketone (TLCK), 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 2.8 Kallikrein Inactivator Units (KIU)/ml aprotinin. The diluted serum was mixed with an equal volume of cell-free translation reaction mixture, and incubated overnight at 4°C. 10 µl of 10% protein-A-Sepharose (Pharmacia, Piscataway, NJ) was added and gently mixed for 1 hour at 4°C. The immune complexes were washed and analyzed on SDS-polyacrylamide gels as described in Dragon et al., Mol. and Biochem., Parasitology, 16:213-229, 1985.

G. Synthesis of cDNA

cDNA was synthesized by methods known to those of ordinary skill in the art. Briefly, 2 µg of epimastigote or trypomastigote A+ mRNA was transcribed by the action of AMV reverse transcriptase as described by Ullrich et al., Science, 196:1313-1319, (1977) and Gubler, Gene, 25:263-269, (1983). Transcription was initiated at the 3' polyadenylated end of the mRNA using oligo(dt) as a primer. The second strand was copied using DNA polymerase I and RNAse H (Boehringer-Mannehim. Indianapolis, IN) and appropriate buffers.

Specifically, 2 µg of oligo-dT (12-18 nucleotides, Pharmacia Molecular Biology Division, Piscataway, NJ) was annealed to 2 micrograms of purified mRNA in the presence of 50 mM NaCl. The annealing reaction was heated to 90°C and then slowly cooled. For the reverse transcriptase reaction, deoxynucleosidetriphosphates (dATP, dTTP, dGTP and dCTP) were added to make a final concentration of 0.5 mM, along with 40 units of enzyme (Molecular Genetic Resources, Tampa, FL). The reverse transcriptase reaction buffer contained 15 mM Tris-HCl, pH 8.3, 21 mM KCl, 8 mM $MgCl_2$, 0.1 mM EDTA. and 30 mM beta-mercaptoethanol. This mixture was incubated at 42°C

for 45 minutes. The RNA-DNA duplex was extracted once with phenol chloroform and then precipitated with ethanol. The pelleted material was then resuspended in 100 microliter reaction mixture containing the following: 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM KCl and 250 uM each dATP, dCTP, dTTP, dGTP.

RNAase H (100 units/ml Pharmacia Molecular Biology Division, Piscataway, NJ) and DNA Polymerase I -- Klenow fragment (50 units/ml Boehringer Mannheim, Indianapolis, IN) were added and the reaction was incubated at 12°C for 60 minutes. The combined activities of these enzymes result in the displacement of the mRNA from the RNA-DNA duplex as the first cDNA strand is used as a template for synthesis of the second cDNA strand. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and the DNA duplex was then extracted with phenol: chloroform and ethanol precipitated. The sequence of the reactions of DNA Polymerase I and RNAase H was predicted to yield cDNA molecules which were blunt ended at both their 3' and 5' ends. A 3' blunt end is necessary for the subsequent cloning of the cDNA.

H. Construction of the cDNA Library

Briefly, the double stranded cDNA preparations were digested with the restriction endonucleases SacI and PvuII (New England Biolabs, Beverly, MA) and ligated, using T4 DNA ligase, into the SacI and SmaI sites of the plasmid pUC18 (Yanish-Perron et al., Gene, 33:103-119, 1985). This mixture was used to transform E. coli K12 strain JM83, selecting for ampicillin resistance conferred by the introduction of the pUC18 into the host cell. From 2 ug of mRNA approximately 150 ng of cDNA were prepared which yielded about 7000 ampicillin resistant clones.

More specifically, the cDNA was resuspended in 100 microliters of sterile water. Approximately 50 ng was digested with SacI (5000 units/ml) and pVUII (12000 units/ml) in the presence of 6 mM Tris-HCl (pH and 6 mM beta-mercaptoethanol for 60 7.4) 6 mM MgCl2' minutes at 37°C.

The sample was then re-extracted with phenol: chloroform and ethanol precipitated. For the cloning step a pUC18 vector was used. The vector had been digested with SacI and SmaI. SmaI provided the blunt end site necessary for ligation of the 3' end of the cDNA. The ligation reaction was performed using 40 ng of vector DNA and 50 ng of cDNA. Ligation was done overnight at 12°C in a ligase buffer of 50 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 20 mM dithiothreitol, 1.0 mM rATP using one unit of T4 DNA ligase.

The recombinant DNA molecules were then introduced into E. coli K-12 strain JM83 by transformation. The transformed bacteria were spread on agar plates containing the antibiotic ampicillin at a concentration of 50 micrograms/ml. Since the plasmid pUC18 contains the ampicillin resistance gene, only those bacteria which acquired a recombinant plasmid survived. These bacteria each grew and divided to form a bacterial colony. Each cell in the colony is a descendant of the original parental cell and contains the same recombinant plasmid. Using hybridization - selection/translation and immunoprecipitation techniques to screen the cDNA library a clone was identified which contained nucleotide sequences corresponding to a 70 kd T. cruzi peptide.

I. Isolation of the full length 70 kd gene

The cDNA clone was used as a probe to screen the T. cruzi Sau3a partial genomic library as described by Maniatis et al. A lambda phage designated FG21 was identified which contained multiple copies of the 70 kD

gene. A 2.4 kb SmaI fragment was sub-cloned into pUC9 from FG 21. This subclone called pEG22 contained one full length copy of the 70 kD gene. The DNA sequence of PEG22 was determined. FG21, was sequenced and used to construct an expression plasmid to allow production of the 70 kd antigen in E. coli.

J. Expression of Cloned Genes in E. coli

Several systems are available in the laboratory for expressions of foreign genes in E. coli and other mammalian and bacterial tissue culture cell lines. It is important to provide the cloned genes with an E. coli ribosome binding site for initiation of translation and a strong promotor to obtain sufficiently high levels of protein. Although obtaining "direct" expression of the protein is possible, it appears to be more efficient to produce the protein as a fusion protein, the amino terminus of which is a small part of an E. coli protein containing signals for the initiation of protein synthesis. The amino terminus of B-lactamase and the amino terminus of B-galactosidase can make such fusion proteins [Hegpeth et al., Mol. Genet., 163:197-203 (1980) and Lingappa et al., PNAS, 81:456-460 (1984)]. These and other systems may be used to obtain expression of the cloned gene.

Sequencing analysis showed that the coding region of the 70 kd gene was flanked by an AhaIII site 30 base pairs upstream from the putative ATG start codon. An additional AhaIII site is located 367 base pairs following the TGA stop codon in the nucleotide sequence of FG21. Subsequently FG21 was digested with the restriction enzyme AhaIII. The resulting DNA fragment was 2,341 base pairs long. It was gel purified and cloned in the SmaI site of the expression vector pUC9. The resulting plasmid, pFP70-47, was used to transform E. coli K12 SG936 bacteria.

A sample of this recombinant bacteria has been placed on deposit with the American Type Culture Collection (12301 Parklawn Drive, Rockville, Maryland, USA) as ATCC number 67254. The culture was deposited on November 4, 1986. This strain, SG936/FP70-47 produces a 70 kd polypeptide which can react with chagasic sera. Expression of the entire protein, however, provides as many determinants as possible on the target antigen.

K. Antigen Production

The transformed E. coli are grown in liquid culture containing 50 micrograms per ml of ampicillin to enhance plasmid ability. Cultures are harvested at an OD of 2.0 measured at 550 nm. The cells are then pelleted and washed and lysed by freeze/thaw and sonication. A detergent extraction solubilizes most of the remaining polypeptides. The 70 kd expressed product, however, remains insoluble and is harvested by centrifugation. This insoluble "cement" is denatured in urea and subsequently diluted at a high pH and the pH is then adjusted back to neutral. During the renaturation process the antigen refolds and achieves that immunologically active conformation. The details of this procedure used are identical to those used to restore enzyme activity to recombinant chymosin as described by McCaman et al., J. Biotech., 12:117-191, (1985).

Example 2 -- 74.5 kda M. Hyo Antigen and Use As a Vaccine

A. Preparation of M. hyopneumoniae DNA

Strain P-57223 (obtained from Dr. Charles Armstrong, Purdue University) was grown in 1 liter of Friis medium to a density of approximately 10^9 to 10^{10} color changing units per ml. The cells were harvested by centrifugation and resuspended in 2 ml

phosphate buffered saline which brought the total volume to 3.25 ml. The suspension was then mixed with a solution consisting of 24.53 g cesium chloride dissolved in 19.75 ml 10 mM Tris pH 8.0 1 mM EDTA and 1.53 of 10 mg/ml ethidium bromide was added. This was mixed with a solution consisting of 3.87 g cesium chloride dissolved in 2.15 ml 10 mM Tris pH 8.0. 1 mM EDTA, 8.9% Sarkosyl. The resulting suspension was incubated at 65°C for 10 minutes to completely lyse the cells. The DNA was separated by equilibrium buoyant density centrifugation in a Sorvall TV850 rotor at 43,000 rpm for 18 hours, and withdrawn with an 18 gauge needle. This DNA was subjected to two additional buoyant density centrifugations in a Sorvall TV865 rotor at 55,000 rpm for 7 and 18 hours respectively, each time the band of genomic DNA being removed with an 18 gauge needle. The resulting DNA solution was extracted with cesium chloride saturated isopropanol, to remove ethidium bromide, and extensively dialyzed against 10 mM Tris pH 8.0, 1mM EDTA, to remove the isopropanol and cesium chloride.

B. DNA Probing of M. hyopneumonia DNA

Plasmid pEG22, described in Example 1 is purified from E. coli by methods in the art, and labeled with ^{32}p by nick translation using DNA polymerase I.

pEG22 is used as a probe as follows:

Mycoplasma genomic DNA was digested with EcoRI under the following conditions at 37°C for 2 hours.

114 microliters P-5722-3 DNA
6 microliters H_2O
15 microliters 10X BRL-3 (Bethesda Research Labs)
15 microliters EcoRI (Bethesda Research Labs)

67 microliters were mixed with 0.1% Bromphenol blue, glycerol, loaded onto a 1% agarose gel and electrophoresed until the blue color had migrated to within 1cm of gel end. The DNA was transferred to a nitrocellulose filter by Southern's technigue. The filter was hybridized to the DNA probe described above under conditions which allow hybridization in the absence of exact sequence identity.

Hybridization:

6 X NET
5 x Denhardts solution
2 X 10^6 counts per minute probe,
37°C for 18 hours

Wash:

6 X NET
0.1% SDS
3 times at room temperature,
1 time at 50°C

6 X NET

1 M NaCl
90 mM Tris pH 7.6
6 mM EDTA

Southern blot analysis shows that the DNA probe hybridized to a specific EcoRI restriction endonuclease fragment of approximately 6 kB in length and thus include the antigen's gene.

C. Cloning the Gene by Hybridization

In order to identify the gene by hybridization to the pEG22 DNA probe, 200 micrograms of P-57223 DNA was digested with 120 units of EcoRI in a volume of 600 microliters. The digestion mixture was mixed with glycerol and xylene cyanol blue FF and electrophoresed on a 3.25% acrylamide gel. Five

slices of approximately 0.5 cm were cut from the gel in the size range desired and electroeluted in 0.1% SDS, 0.5 X TBE buffer. The resulting DNA fractions were extracted with phenol/chloroform, ethanol precipitated, and each resuspended in 50 microliters of 10mM Tris pH 8.0, 1mM EDTA. By dot-blot analysis, (See Nuc. Acid Res. 7:1541-1552, 1979), fraction 4 was shown to contain the DNA fragment of interest.

To create a gene library enriched for the desired fragment, 7 microliters of Fraction 4 was ligated to EcoRI digested pUC9 with T4 ligase one-half of the reaction was transformed into JM83 and plated on X-gal plates where white colonies contain plasmids and inserts. Plasmid DNA from 24 white colonies was prepared and transferred to nitrocellulose by the slot-blot modification of the dot-blot procedure and probed with ^{32}P labeled pEG22.

Plasmid DNA preparations which hybridize to the DNA probe are subjected to EcoRI digest analysis to show that each plasmid contains the same size insert fragment, and most likely the same gene. A plasmid is selected for DNA sequence analysis which shows greater than 50% identity to pEG22.

D. Preparation of Genomic Library

A preparative digest of 200 μg genomic DNA of Mycoplasma hyopneumoniae P-57223 was done using 200 units of EcoRI in a total volume of 1 ml and 250 μl aliquots were removed at 6 min, 25 min, 42 min and 63 min.

The four preparative samples of partially digested Mycoplasma DNA were then combined (200 μg) and loaded onto an exponential sucrose gradient. The gradient was centrifuged in a Sorvall AH627 rotor at 26 k rpm for 21 hrs at 15°C.

The gradient was then slowly fractioned from the bottom by collecting 15 drop fractions (90

fractions total). 20 µl of each fraction was then run on a 1% agarose gel as described above. Fractions containing DNA fragments smaller than 18 kbp and larger than 15 kbp were pooled (fractions 32-40) and dialyzed against TE (10 mM Tris.HCl pH 7.5, 1 mM EDTA pH 8.0) to remove the sucrose. The DNA (3.5ml) was then precipitated with ethanol and resuspended to about 15 µl (1 mg/ml) under vacuum and stored at -20°C.

EcoRI Arms of bacteriophage lambda-Dash were obtained from Vector Cloning Systems (StrataGene) and were ligated at a concentration of 200 µg/ml to Mycoplasma target DNA at a concentration of 25 µg/ml in a total volume of 10 µl using T4 ligase (Boehringer GmbH) at a concentration of 100 units/ml. The ligation reaction was incubated at room temperature for 2 hours. 4 µl of the ligation was then packaged into lambda particles using the in vitro packaging kit Gigapack (StrataGene). The phage was then titered on E. coli strain P2392 (StrataGene) and found to be 7.75×10^5 pfu/ml (3.1×10^5 pfu/ug of lambda-Dash).

E. Screening of Library

The library is screened using the plasmid previously obtained which shows greater than 50% homology to pEG22, by the previously described probing procedure. DNA from positive recombinants is prepared, digested with EcoRI, analyzed by gel electrophoresis, to indicate portions of the M. hyopneumoniae genome composed of several EcoRI restriction fragments. One of the fragments is digested with EcoRI, ligated to EcoRI digested pWHA148 and transformed into E. coli strain JM83 and called pMYCO16; its DNA was prepared and digested with a number of different restriction endonucleases in order to derive the restriction map shown in Figure 3.

Plasmid pWHA148 is prepared by inserting a synthetic oligonucleotide into the Hind III site of pUC18. The amino terminal coding sequence of the X-complementing peptide of B-galactosidase is shown in Figure 4, and contains 8 additional restriction sites over the parent pUC18. The oligonucleotide insert into pUC18 is shown in Figure 4 between the Sph1 and Hind III sites.

An N-terminal portion of pEG22 is used by Southern analysis to hybridize to the 0.6kb AccI-AsuII restriction fragment of pMYC016. DNA sequence analysis of the 0.6 kb fragment identifies that start codon of the homologous gene.

On the restriction map of pMYC016 (Figure 3 the gene begins within the 0.6 kb AccI-AsuII restriction fragment, extends clockwise within the 0.4 kb AsuII - ClaI, 1.2 kb ClaI - ClaI, and 1.4 kb ClaI-HindIII fragments, and ends short of the HindIII site. DNA sequence analysis shows that pMYC016 contains a 74.5 kD protein homologous to the 70 kD T. cruzi heat shock antigen.

The DNA-amino acid sequence of the 74.5 kD gene is shown in Figure 5.

F. Expression of full length M.hyo. 74.5 kD antigen in E. Coli

Plasmid pMYC016 DNA (Figure 3) was digested with AccI, treated with Mung Bean nuclease to remove the single stranded AccI tails, re-ligated to delete the 1.9 kb AccI fragment in front of the 74.5 kD antigen gene and transformed into E. coli strain JM83. One transformant was named pMYC029; its DNA was digested with a number of different restriction endonucleases in order to derive the restriction map shown in Figure 6.

pMYC029 was subjected to DNA sequence analysis which showed that a spontaneous deletion had occured at the ligation juncture, where two bases were deleted and the PstI site was retained, as shown below (only a portion of the 5' to 3' strands are represented).

pMYC029 expected: TTGCATGCCTGCAGGTACTTTCTTTTGTCT
PstI
pMYC029 observed: TTGCATGCCTGCAGGCTTTCTTTTGTCT
PstI

This fortuitous deletion allows the in frame insertion into the pUC9 open reading frame. Plasmid pMYC029 is a low level expression plasmid.

G. Construction of pMYC031 and expression of 74.5 kD antigen fragment

Because the mycoplasma insert of pMYC029 is oriented away from the Lac promoter of pWHA148, it was desired to insert the gene into another expression vector, pUC9. The two base deletion enabled the gene for the 74.5 kD antigen to be placed in the same reading frame as the beta-galactosidase gene of E. coli vector pUC9.

In order to perform this construction, pMYC029 DNA was digested with PstI and EcoRI, the PstI - EcoRI fragment containing the entire 74.5 kD coding sequence was purified, ligated to the PstI and EcoRI digested vector pUC9, and transformed into E. coli strain JM83. One transformant was named pMYC031 (Figure 7); its DNA was prepared and transformed into E. coli strain W3110 by the transformation procedure described above.

H. Construction of pMYCO32

It is known that TGA codons encode the amino acid tryptophan in mycoplasma but normally terminate peptide chain elongation in E. coli and that the trpT176 gene, a mutant tryptophan t-RNA which recognizes UGA (Raftery, et al., Jour. Bacteriol., 158:849-859), allows peptide chain elongation at TGA codons in E. coli laboratory mutants. We reasoned that the addition of trpT176 to expression vectors would allow E. coli peptide chain elongation at the mycoplasma TGA codons of cloned genes.

Plasmid pCAM101 was purchased from James Curran (University of Colorado) as a convenient source of the trpT176 gene and is shown in Figure 8.

DNA from pCAM101 was digested with EcoRI, the 0.3 kb EcoRI fragment which contains the trpT176 gene was purified, ligated to EcoRI digested pMYCO31, and transformed into E. coli strain W3110. One transformant was named pMYCO32 and its restriction map is shown in Figure 9.

I. Expression of M. hyopneumoniae 74.5 kD antigen in E. coli

A W3110 (pMYCO32) transformant was selected, grown in L-broth, lysated as previously described, and a portion subjected to polyacrylamide gel electrophoresis. New 75 kD and 43 kD proteins were identified by gel electrophoresis which represented approximately 5% and 0.1% of total E. coli protein, respectively. The pMYCO32 75 kD protein was shown by Western blot to react with the previously described pig antisera raised against the 74.5 kD M. hyopneumoniae antigen.

An improved expression plasmid pMYCO87 has been deposited with the ATCC on June 30, 1989 as ATCC number 68030. It contains an in vitro change of TGA to TGG (Tryptophane) at codon position 211 (see Figure 5).

J. Use of the recombinant form of Mycoplasma hyopneumoniae 74.5 kD antigen as a vaccine

A W3110 (pMYC032) transformant from Example 2 was selected, grown in M-9 minimal medium in a 14 liter Chemap fermenter to a cell density of 110 O.D. 600, and 120 g (wet weight) of cells were harvested from 500 ml by centrifugation. A suspension was prepared consisting of 2.3 g of cells per 10 ml of PBS containing 12 mM EDTA, 0.5 mg/ml lysozyme. The suspension was incubated at 25 °C for 15 minutes. sonicated on ice for 2 minutes in 30 second bursts, centrifuged at 13,000 g for 10 minutes at 4°C, and the soluble fraction reserved as product. A portion of the product was subjected to polyacrylamide gel electrophoresis. The recombinant form of 74.5 kD antigen made up approximately 25% of the soluble protein and the yield dosages were prepared in PBS at 100 and 500 μg per dose and emulsified on ice with equal volumes of Freund's incomplete adjuvant (Sigma) immediately prior to use.

Vaccination Test

Week 0 Three litters of Hampshire, Hampshire X Duroc, and York piglets taken by Caesarian section.

Week 1 Piglets divided randomly into 7 pig dosage groups and each vaccinated sub-cutaneously in leg.

Week 3 Booster vaccination, as above, opposite leg.

Week 8 Challenge administered by trans-tracheal inoculation of 10^6 CCU Mycoplasma hyopneumoniae.

Week 12 Necropsy of experimental animals and infection controls.

The results were as follows:

Group	Incidence*	Severity**
Control	5/5	12.4 ± 4.7
100 ug 74.5 kD	1/4	4.2 ± 4.9
100 ug recomb. 74.5 kD	2/6	9.7 ± 11.7
500 ug recomb. 74.5 kD	4/4	25.0 ± 6.1

* Number of pigs with a lung lesion score greater than 5%

** % of lung surface effected (mean ± std. dev.)

Example 3. -- The 70 kD Hsp Analog from Mycoplasma Gallisepticum.

A. Preparation of Genomic Libraries

Two strains of M. gallisepticum F-K810 and R, were obtained from R. Yamamoto (U. C. Davis) and grown in F-80 media for the preparation of genomic DNA. (Nord Veterinaermed. 27:337-339).

Approximately 22 ml of stationary phase M. gallisepticum culture was centrifuged at 13,000 X g at 4°C for 10 minutes to harvest mycoplasma cells. The supernatant was discarded and the cell pellet was resuspended in PBS to wash. Cells were harvested by centrifugation after washing. The cells were washed a total of three times with PBS and the resulting cell pellet frozen at -78°C. After thawing, the cells were resuspended in 2 ml 10 mM Tris-HCl pH 8.0, 50 mM EDTA, 1% SDS, and 100 µg Proteinase K was added. The cells were lysed at 50°C for one hour with occasional mixing. The lysate was extracted with phenol then with chloroform/isoamyl alcohol to remove cellular debris. The DNA-containing aqueous phase was dialyzed against 4 liters of 10 mM Tris-HCl, 5 mM EDTA twice, and 10 mM Tris-HCl, 1 mM EDTA once. From each strain, 60 µg of DNA was recovered, an amount sufficient for restriction analyses. Southern blot analyses, and library construction. Restriction digests indicated that the

two strains are similar to each other with limited restriction fragment length polymorphism.

B. Mixed oligonucleotide probes for isolating the Hsp70 protein from M. gallisepticum

When the Hsp70 amino acid sequence from T. Cruzi aligned with the amino acid sequence of the M. hyopneumoniae 74.5 kD antigen of Example 2. Several regions containing sequences six amino acids in length are identical in both sequences. The array of DNA sequences which could encode these amino acid regions was determined. The two amino acid sequences corresponding to nucleotide sequences having the lowest degeneracy, were selected for use as oligonucleotide probes. These were synthesized as follows:

COD1159 Ile-Ile-Asn-Glu-Pro-Thr

ATA	ATA	AAC	GAA	CCA	AC
C	C	T	G	C	
T	T			G	
				T	

COD1218 Gly-Gly-Gly-Thr-Phe-Asp

GGA	GGA	GGA	ACA	TTC	GA
C	C	C	C	T	
G	G	G	G		
T	T	T	T		

Pools of the above oligonucleotides were labeled with ^{32}P using polynucleotide kinase (BRL) and used to probe Southern transfers of HindIII digested M. gallisepticum chromosomal DNA. After 50°C washes in 6X NET, 0.1 SDS, COD 1159 hybridized to two HindIII fragments. COD 1218 hybridized to two HindIII fragments at 45°C under likewise identical conditions. Both probes hybridize to an apparently identical 3.4 kb fragment, where as the other fragments differ in length

and probably represent hybridizatlon due to non-specific sequence homology. The hybridization of both probes to the same 3.4 kb HindIII fragment is highly significant as the probability that hybridization of both probes to the same fragment of genomic DNA results from non-specific sequence homology is less that $2X10^{-3}$. The hybridization patterns for DNA purified from strain R strain and F-K810 strain of M. gallisepticum were identical to one another.

Plasmid DNA from pMYCO87, containing the gene for M. hyopneumoniae (ATCC 68030 deposited with the American Type Culture Collection on June 30, 1989) was labeled using the Boeringer Mannheim nonradioactive Southern hybridization kit (Genius kit) and used to probe a Southern transfer of EcoRI and HindIII restriction digested chromosomal DNA from the F-strain and M. hyopneumoniae as a positive control. The probe detected bands of the expected size in the M. hyopneumoniae genome and an EcoRI band of 6.8 kb and a Hind III band of 3.3kb in the M. gallisepticum digests after washes at 65°C in 0.5X SSC and 0.1% SDS.

C. Preparation of Size Selected Genomic Libraries

The general approach for cloning the hsp antigen gene from M. gallisepticum was analogous to the procedure used for the T. cruzi 70 kD hsp. M. gallisepticum genomic DNA, 1 μg from both the R strain and the F-K8 I 0 strain, was digested to completion with the bacterial restriction endonuclease HindIII and separated on 3.25% polyacrylamide gels. DNA from four gel slices containing restriction digest fragments between 2 and 5 kb was electroeluted. An aliquot of DNA electroeluted from each of the four gel slices was subjected to agarose gel electrophoresis, transfered to a nitrocellulose membrane by Southern transfer and probed with ^{32}P-labeled COD1159 to identify the fraction which contains the 3.3kb hybridizing HindIII band. In

this way, a positive DNA fraction was identified. This positive DNA fraction was then ligated into Hind III digested pUC9 and transformed into E. coli DH5a.

D. Identification of Positive Clones

For each strain, 12 and F-K810, plasmid DNA from forty-eight recombinant clones was isolated by the method of Holms and Quigley 1981 (Anal. Biochem. 114:193-197, 1981), transferred to nitrocellulose using a Bio-Rad dot blot apparatus, and probed with COD1159 in the case of the R-strain or both COD1159 and COD1218 on duplicate blots In the case of strain F-K810.

One positive isolate was found for each strain. Plasmid pMGA4 contains a positive R-strain insert and has been deposited with the American Type Culture Collection on _____1989 with the designation _____. A map of pMGA4 is provided in Figure 10. The sequence of of the M. gallispeticum Hsp70 DNA and the derived amino acid sequence is provided in Figure 11.

E. Expression, Purification and Use as a Vaccine

DNA from pCAM101 was digested with EcoRI, a 0.3 kb EcoRI fragment including trpT176 was purified, ligated to EcoRI digested pUC9, transformed into E. coli strain JM83, and one transformant was named pWHA160 (see Figure 12).

Plasmid pMGA4 DNA was digested with HindIII and BglII, ligated to HindIII and BamHI digested pWHA160, digested with BamHI and BgIII, and transformed into E. coli strain DH5a. One transformant was named pMGA10. The MGA10 transformant was grown in L-broth at 37°C, and the cells harvested by centrifugation and frozen. The cell pellet from 4 ml of culture was resuspended in 100 µl of a solution consisting of 0.5 mg/ml hen egg-white lysozyme dissolved in 25 mM Tris pH 8.0 10 mM EDTA; and incubated at 25°C for 10 minutes.

A portion of the resulting lysate was subjected to polyacrylamide gel electrophoresis and a new 67 kD protein was identified. Western blot analysis, using pig anti-74.5kD serum, showed that the new 67 kD protein was immunologically related to Hsp70.

F. Use of Bacterially Produced M. gallisepticum Hsp 70 Protein to Raise an Immune Response in Chicken

The purified M. gallisepticum protein is concentrated by lyophilization and resuspended to a final concentration of 0.5-2.0 mg/ml in 0.1% SDS. For use, the immunizing antigen is formulated in one volume of protein concentrate to three volumes of oil carrier consisting of 5% Arlacel, 94% Drakeol 6-VR and 1% Tween 80. The dose of the antigen employed is 100 μg/dose. Chicken receive the formulated vaccine by subcutaneous injection. A booster vaccination by the same route is done two weeks later.

Numerous modifications and variations of the present invention are possible in light of the above teachings; therefore, within the scope of the appended claims the invention may be practiced otherwise than as particularly described.

Table 1. Representative Pathogenic Organisms.

1: DISEASE AGENTS
1.1: BACTERIA
1.1.1: ACTINOBACILLUS SPP.
1.1.1.1: Actinobacillus lingiresii
Mastitis infections in cattle, sheep, swine, equine
1.1.1.2: Also known as Haemophilus
swine pneumonia

1.1.2: BACILLUS SPP.
Bacillus anthracis
Anthrax, an acute febrile disease of all mammals

1.1.3: BORDETELLA SPP.
1.1.3.1: B. bronchiseptica - repiratory disease in many species
1.1.3.2: B. pertussis - whooping cough in man
1.1.4: BORRELIA SPP.
1.1.4.1: B. burgdorferi - Lyme disease in dogs, deer, man
1.1.5: BRUCELLA SPP.
1.1.5.1: Brucella abortus, B. suis, B. melitensis
brucellosis in cattle, sheep, swine, equine, canine, man

1.1.6: CAMPYLOBACTER SPP.
1.1.6.1: Campylobacter fetus
causes infertility and embryonic death in cattle, swine, sheep, equine
(vibriosis)
1.1.6.2: Vibrio cholerae - cholera in man

1.1.7: CHLAMYDIA SPP.
1.1.7.1: C. psittaci - respiratory disease in birds
1.1.7.2: C. cati - conjunctivitis in cats

1.1.8: CLOSTRIDIUM SPP.
1.1.8.1.: C. chauvoei
blackleg in cattle and sheep
1.1.8.2: C. septicum
malignant edema in cattle and sheep
1.1.8.3: C. haemolyticum
red water in cattle
1.1.8.4: C. novyi
black disease in cattle and sheep
1.1.8.5: C. sordelli
big head disease in cattle and sheep
1.1.8.6: C. perfringens
enterotoxemia in cattle, sheep, swine, equine, gas gangrene in man

1.1.8.7: C. tetani
tetanus in all mammals
1.1.8.8: C. boutulinum
8 types, causing botulism in all species

1.1.9: CORYNEBACTERIUM SPP.
1.1.9.1: C. diptheria - Diptheria in man
1.1.9.2: C. pyogenes -causes pyogenic processes in cattle, sheep, swine, goats
1.1.9.3: C. renale - cystitis in cattle
1.1.9.4: C. equi - pneumonia in horses

1.1.10.1: ERYSIPELOTHRIX SPP.
1.1.10.1: Erysipelothrix rhusipothiae - erysipelas in swine and man

1.1.11: HAEMOPHILUS SPP.
1.1.11.1: H. influenza, respiratory disease in various species
1.1.11.2: H. paraninfluenza, H. parasuis, H. suis - respiratory disease in swine

1.1.12: KLEBSIELLA SPP.
1.1.12.1: Klebsiella pneumoniae - Pneumonia and septicemia in animals and man

1.1.13: LISTERIA SPP.
1.1.13.1: L. monocytogenes - Listeriosis -- encephalitis in ruminants

1.1.14: MYCOBACTERIUM SPP.
1.1.14.1: M. tuberculosis, M. bovis, M. avium - Tuberculosis in various species
1.1.14.2: M. paratuberculosis - Johne's disease in cattle, sheep, and goats

1.1.15: PASTEURELLA SPP.
1.1.15.1: P. pestis - Plague in man and rodents
1.1.15.2: P. haemolytica, P. multocida - respiratory disease in many species

1.1.16: PSEUDOMONAS SPP.
1.1.16.1: P. aeruginosa - respiratory disease in various animals
1.1.16.2: P. mallei - Glanders disease in dogs and cats

1.1.17: SALMONELLA SPP.
1.1.17.1: S. typhimurium - enteric disease in a number of species
1.1.17.2: S. typhisuis, S. choleraesuis - enteric disease in swine
1.1.17.3: S. typhi - Typhoid fever

1.1.17.4: S. paratyphi - Paratyphoid - A in man
1.1.17.5: S. gallinarum - fowl typhoid
1.1.17.6: S. pullorum - pullorum disease in chickens

1.1.18: STREPTOCOCCUS SPP.
1.1.18.1: S. agalactiae, S. dysgalactiae - mastitis in numerous species
1.1.18.2: S. dispar - enteritis in numerous species
1.1.18.3: S. equi - cholic in horses
1.1.18.4: S. genitalium - uterine infections in horses
1.1.18.5: S. pneumoniae - respiratory disease in man

1.1.19: STAPHYLOCCUS SPP.
1.1.19.1: S. aureus - mastitis in many species
1.1.19.2: S. epidermidis - pyoderma in many species

1.1.20: TULAREMIA SPP.
1.1.20.1: Francisella tularensis - Tularemia in man

1.2.6: HERPESVIRIDAE
1.2.6.1: H. simplex Type 1 - Oral Herpes in man
1.2.6.2: H. simplex Type 2 - Genital Herpes in man
1.2.6.3: Epstein-Barr Virus - Mononucleosis in man
1.2.6.4: H. smiae - Herpes B. in primates
1.2.6.5: H. suis-Adjuskie's disease - pseudorabies in swine and cattle
1.2.6.6.: H. canis - Respiratory infection of dogs
1.2.6.7: H. equi - Equine rhinopneumonitis - respiratory and abortion in horses
1.2.6.8: H. bovis - IBR (Infectious Bovine Rhinotracheitis) in cattle
1.2.6.9: H. felis - FVR (Feline Viral Rhinotracheitis)
1.2.6.10: Laryngotracheitis virus - Laryngotrachetis in birds
1.2.6.11: Marek's Disease Virus - Merek's disease in birds
Feline calicivirus (FCV)
1.2.6.12: Cytomegaloviruses-many diseases in various animals

1.2.13: POXVIRIDAE
1.2.13.1: SMALLPOX - WAS A MAJOR DISEASE IN MAN
1.2.13.2: VACCINIA - USED TO VACCINATE AGAINST SMALLPOX
1.2.13.3: COWPOX - SKIN DISEASE OF CATTLE
1.2.13.4: SWINEPOX - SKIN DISEASE OF SWINE
1.2.13.5: ECTROMELIA - MOUSEPOX
1.2.13.6: AVIPOXVIRUSES - FOWLPOX, CANARYPOX, PIEGEONPOX, TURKEYPOX,

1.2.13.7: CAPRIPOXIVIRUSES - LUMPY SKIN DISEASE IN SHEEP AND GOATS
1:2:13:8: PARAPOXIVIRUSES - "SORE MOUTH" IN SHEEP AND GOATS, BOVINE PAPULAR STOMATITIS

1.3: MYCOPLASMA
1.3.1: M. mycoides - Bovine respiratory disease
1.3.2: M. bovis - bovine mastitis
1.3.3: M. bovigenitalium - bovine epidymitis
1.3.4: M. bovoculi - Infectious bovine keratoconjuntivitis
1.3.5: M. bovirhinis and M. dispar - respiratory disease
1.3.6: M. hyorhinis and M. hyosynoviae - respiratory disease and lameness in swine
1.3.7: m. gallisepticum and M. synoviae - respiratory disease in poultry

1.4: RICKETTSIA
1.4.1: Rickettsiaceae
1.4.1.1: R. prowazekii - Typhus fever
1.4.1.2: R. typhi - murine thyphus in man
1.4.1.3: R. rickettsii - Rocky Mountain Spotted Fever
1.4.1.4: Coxiella Burnetii - Q Fever in cattle, sheep, goats, birds, and man
1.4.1.5: Cowdria ruminatum - Heartwater in cattle
1.4.2: Anaplasmataceae
1.4.2.1: A. marginale and A. centrale - Anaplasmosis in cattle
1.4.2.2: A. ovis - Anaplasmosis in sheep
1.4.2.3: Haemobartonella felis - Hemobartonellosis in cats (Feline Infectious Anemia)
1.4.2.4: Haemobartonella canis - Hemobartonellosis in dogs
1.4.2.5: Eperythrozoon - parasites which attack red blood cells in various animals

1.5: CHLAMYDIACEAE
1.5.1: C. psittaci - Psittacosis - a febrile pulonary disease in man and birds
1.5.1.1: also causes Sporadic Bovine Encephalomyelitis and polyarthritis in cattle
1.5.1.2: also causes Epizootic Abortion in cattle and sheep
1.5.1.3: also causes pneumonia in cattle and hseep
1.5.1.4: also causes Feline Pneumonitis in cats
1.5.2: C. trachomatis - Veneral disease in man

1.6: SPIROCHAETALE
1.6.1: Leptospria spp.

1.6.1.1: L. canicola, L. grippotyphosa, L. hardjo, L. icterohaaemorrhagiae
1.6.1.2: L. pomona - all cause disease in various species
1.6.2: Treponema SPP.
1.6.2.1: T. hyodysenteriae - Swine Dysentery
1.6.2.2: T. pallidum - Syphilis in man
1.6.3: Borrelia spp.
1.6.3.1: B. anserina 0 Avian borrelosis or spirochaetosis in birds

1.7: FUNGAL DISEASES
1.7.1: Asperigillus fumigatus - brooder pneumonia in poultry
1.7.2: Blastomyces dermatitidis - pulmonary infection in animals and man
1.7.3: Candida albicans - Thrush in birds, cats, cattle, swine and man
1.7.4: EPIDERMOPHYTON SPP.
1.7.4.1: E. floccosum - Athletes foot in man
1.7.5: HISTOPLASMA SPP.
1.7.5.1: H. capsulatum - systemic fungal infection in many species
1.7.6: MICROSPORUM SPP.
1.7.6.1: M. canis - ringworm in dogs, cats, man, cattle
1.7.6.2: M. gypseum - ringworm in dogs, cats, horses, man
1.7.7: TRICHOPHYTON SPP.
1.7.7.1: T. rubrum - ringworm in dogs, primates, and man
1.7.7.2: T. equinum and T. quinkeanum - ringworm in horses
1.7.8: MYCOTOXICOSES (Moldy feed) caused by numerous filamentous fungi
1.7.8.1: Aflatoxins, Mycotoxins, Aspergillus toxins

2: PARASITES
2.1: PROTOZOA
2.1.1: AMEBA
2.1.1.1: Entamoeba histolytica - Amebic dysentery in dogs, cats, pigs and man
2.1.2: BABESIA SPP.
2.1.2.1: Babesia bigemina and B. bovis are major causes babesiosis in cattle (babesiosis also known as Texas fever, Tick Feber, Prioplasmosis)
2.1.2.2: B. argentina, B. Divergens, and B. major also cause babesiosis in cattle
2.1.2.3: B. canis and B. Gigsoni - cause babesiosis in dogs

2.1.2.4: B. equi and B. caballi cause babesiosis in horses
2.1.2.5: B. motasi and B. ovis - cause babesiosis in horses
2.1.2.6: B. trautmanni - babesiosis in pigs
2.1.2.7: B. felis - babesiosis in cats

2.1.3: COCCIDIA
2.1.3.1: EIMERIA SPP.
E. tenelia, E. necatrix, E. brunetti, E. acervulina, E. maxima in chickens
E. bovis, E. zuernii in cattle
2.1.3.2: ISOSPORA SPP.
I. suis - seine
2.1.3.3: SARCOYSTIS SPP.
S. tenella - infects sheep
S. blanchardi, S. fayerei, and S. fusiformis - infect cattle
S. miescheriana - infects swine
2.1.3.4: TOXOPLASMA GONDII
wide spread distribution, especially in cats, swine, sheep, humans
causes abortion, birth defects, deafness
2.1.3.5: CRYTOSPORIDUM SPP.
cause diarrhea in cattle, swine, sheep, birds, and man
A component of AIDS complex
2.1.4: GIARDIA SPP.
2.1.4.1: G. lamblia - infects man
2.1.4.2: G. canis - infects dogs
2.1.4.3: G. cati - infects catas
2.1.4.4: G. bovis - infects cattle
2.1.5: LEISHMANIA SPP.
2.1.5.1: L. donovani - visceral leishmania in man, dogs, cats, cattle sheep
2.1.5.2: L. tropica - cutaneous leshmania in man, dogs, and rodents
2.1.5.3: L. braziliensis - American leishmaniasis in man, dogs, and cats
2.1.6: PLASMODIUM SPP.
2.1.6.1: Plasmodium falciparum - malaria in man
2.1.6.2: P. malariae, P. vivax, and P. ovale - malaria in man
2.1.6.3: P. gallinaceum - avian malaria
2.1.6.4: numerous Plasmodium spp. cause malaria in man
2.1.7: PNEUMOCYSTOSIS SPP.
2.1.7.1: P. carinii - cause of pneumonia in man, dogs, horses, swine, goats
2.1.7.2: A component of the AIDS complex
2.1.8: THEILERIA SPP.
2.1.8.1: T. parva, T. annulata, T. mutans, T. lawrencei and T. cervi

all cause East Coast Fever in cattle, buffalo and deer

2.1.8.2: T. hirci and T. ovis infect sheep

2.1.9: TRITRICHOMONAS SPP.

2.1.9.1: T. vaginalis - a veneral disease of man

2.1.9.2: T. foetus - causes trichomonaiasis, a genital infection of cattle

2.1.9.3: Trichomonas gallinae - causes tricomoniasis, a G.I. infection in birds

2.1.10: TRYPANOSOMA SPP.

2.1.10.1: T. cruzi - Chagas disease in man

2.1.10.2: T. congolense -- Trypanosomiasis in cattle, horses, pigs, dogs

2.1.10.3: T. rhodesiense and T. gambiense - sleeping sickness in man and antelope

2.2: HELMINTHS

2.2.1: TREMATODES

2.2.1.1: FLUKES

Fasciola hepatica - cattle and sheep

F. gigantica - cattle and sheep

Fascioloides magna - cattle, sheep and swine

Dicrocoelium dendriticum - cattle, sheep, horses, swine, man

2.2.1.2: SCHISTOSOMIASIS

Schistosoma japonicum, S. hematobium, S. mansoni, S. intercalatum - man

S. bovis, S. spindale, S. mattheei - cattle, sheep, goat, horse

S. nasalis, S. indium - cattle, sheep, goats

2.2.1.3: PARAGONIMIASIS (SALMON POISONING)

Paragonimus westermani - man

P. kellicotti - mink, dog, cat, pig

2.2.2: CESTODES

2.2.2.1: TAPEWORMS

Taenia saginata, and T. solium - man (cysticercus)

Echinococcus granulosus, and E. multilocularis - man, dog

Taenia hydatigena, T. ovis - dog

T. pisiformis - dog and cat

Dipylidium caninum - dog and cat

Anoplocephala magna, A. perfoliata - horses

2.2.2.2: ECHINOCCUS SPP.

2.2.2.3: DIPHYLLOBOTHRIUM SPP.

2.2.2.4: SPIROMETRA SPP.

2.2.2.5: FASCIOLA SPP.

2.2.3: NEMATODES

2.2.3.1: FILARIAL PARASITES

Dirofilaria immitis - heartworm in dogs

2.2.3.2: HOOKWORMS

A. duodenale and Necator americanus - hookworm in man
A. caninum, A. braziliense - dogs and cats
Uncinaria stenocephala - dogs
Bunostomum phlebotomum - cattle
B. trigonocephalum - sheep and goats
Globecephalus urosubulatus - swine

2.2.3.3: KIDNEY WORMS
Dicoctophyma renale - dog

2.2.3.4: LUNGWORMS
Dictyocaulus viviparus - lungworm in cattle
D. filaria - lungworm in sheep, goat, cattle
Muellerium capillaris - lungworm in sheep
Metastrongylus apri, M. pudendotectus, M. salmi - swine

2.2.3.5: NODULAR WORMS
Oesophagostomum denatum - swine
O. radiatium, and O. columbianum - cattle, sheep, goats

2.2.3.6: ONCHOCERIASIS
Onchocerca volvulus - blindness in humans

2.2.3.7: PINWORMS
Enterobius vermicularis - man
Oxyuris equi - horses
Skrjabinema ovis - sheep and goats

2.2.3.8: ROUNDWORMS
Ascaris lumricoides - roundworms in man, swine
Toxocara canis - dogs
Toxocara cati - cats
Parascaris equorum - horse
Ascaridia galli - chickens

2.2.3.9: SPIROCERCAS
Spriocerca lupi - dogs

2.2.3.10: STOMACH WORMS
Habronema, H. majus, H. megastoma - horses

2.2.3.11: STRONGYLES
Strongylus vulgaris, S. equinus, S. edentatus - horses

2.2.3.12: STRONGYLOIDS
Strongyloides westeri - horses
S. stercoralis - man
S. ransomi - swine
S. canis - dogs
S. tumefaciens - cats

2.2.3.13: TRICHINA
Trichinella spiralis - trichinella in swine and man

2.2.3.14: TRICHOSTRONGYLES

Ostertagia ostertagi - cattle
Haemonchus placei - cattle
Trichostronglyus axei - cattle
Cooperia punctata - cattle
Haemonchus contortus, Cuperia curticei - sheep
Ostertagia circumcincta - sheep
Trichostronglyus colubriformis - equine, swine, cattle, sheep
Nematodirus filicollis - cattle and sheep
Hyostrongylus rubidus - swine

2.2.3.15: WHIPWORMS
Trichuris ovis - cattle, sheep, goats
Trichuris suis - swine
T.. trichiura - man
T. vulpis - dogs

2.3: ARTHROPODS

2.3.1: ACARIASIS

2.3.1.1: Demodex folliculorum - mange in dogs, cats, cattle, swine, sheep, man

2.3.1.2: Demodex phylloides - mange in swine

2.3.1.3: Dermacentor andersoni - wood tick

2.3.1.4: Dermanyssus gallinae - red mite in poultry

2.3.1.5: Ixodes holocyclus - Austrailian tick

2.3.1.6: Notoedres cati - cat mange

2.3.1.7: Otobius megnini - spinose ear tick

2.3.1.8: Ostodectes cynotis - ear mite in dog, cat

2.3.1.9: Psoroptes communis - scab in cattle, sheep, horses

2.3.1.10: Sarcoptes scabiei, S. canis - mange in dogs

2.3.2: DIPTERA

2.3.2.1: BOTFLIES
Gasterophilus intestinalis - equine botfly
Gasterophilus hemorrhoidalis - equine nose botfly
Gasterophilus nasalis - equine chinfly
Gasterophilus pecorum - European botfly
Gasterophilus inermis - botfly
Oestrus ovis - sheep botfly

2.3.2.2: FLEAS
Otenocephalides canis - dog flea
Ctenocephalides felis - cat flea

2.3.2.3: FLIES
Chrysops spp. - deer flies
Fannia spp. - little house flies
Haematobia irritans - horn flies
Haematotobia irritans exigua - buffalo fly (similar to horn fly)
Hermetia illucens - black soldier fly
Hybomitra spp. common fly

Hydrotaea irritans - head flies
Ophyra spp. - dump flies
Melophagus ovinus - sheep ked
Musca autumnalis - face flies
Musca domestica - house fly
Muscina spp. - false stable flies
Simulium spp. - black flies (no-see-ums)
Stomoxys calcitrans - stable flies
Tabanus spp. - horse files

2.3.2.4: GRUBS
Hypoderma lineatum, H. bovis - Heel fly, cattle grub
Calitroga americana - screw-worm fly
Dermatobia hominis - cutaneous myiasis in man, cattle sheep, dogs, cats
Cochliomyia hominivorax - blow fly

2.3.2.5: LICE
Damalinia bovis - cattle biting louse
Anoplura spp. - cattle louse
Haematopinus eurysternus - shortnosed cattle louse
Linognathus vituli - longnosed cattle louse
Solenoptes capillatus - little blue cattle louse
Haematopinus suis - swine lice
Haematopinus asini - horse sucking louse
Trichodectes canis - dog louse
Felicola subrostrata - cat louse

2.3.2.6: MOSQUITOES
Aedes spp.
Anopheles spp.
Culex spp.
Culiseta spp.
Psorophora spp.

Disease	Pathogen(s)
Malaria	Plasmodium falciparum P. vivax P. malariae P. ovale P. berghei etc.
Chagas' Disease	Trypanosoma cruzi
African Trypanosomiasis	Trypanosoma gambiense T. rhodesiense T. brucei etc.
Leishmaniasis	Leishmania donovani

	L. infantum L. tropica L. mexicana L. braziliensis L. chagasi etc.
Leprosy	Mycobacterium leprae
Tuberculosis	Mycobacterium tuberculosis
Filariasis	Brugia malayi B. timori Onchocerca volvulus Wuchereria bancrofti
Schistosomiasis	Schistosoma mansoni S. japonicum
Leptospirosis	Leptospira interrogans L. iceterohaemorrhagiae L. hebdomadis L. pomona etc.
Plague	Yersinia pestis
Typhoid Fever	Salmonella typi
Cholera	Vibrio cholerae
Diptheria	Corynebacterium diphtheriae
Lyme Disease	Borrelia burgdorferi
Pneumonia/bronchitis	Streptococcus pneumoniae Mycoplasma pneumoniae Branhamella catarrhalis Bordetella bronchiseptica Haemophilus influenza
Urethritis	Mycoplasma hominis Ureasplama urealyticum
Giardia	Giardia lamblia
Amoebic dynsentery	Entamoeba histolytica
Syphilis	Treponema pallidum
Chlamydia	Chlamydia trachomatis
Candidiasis	Candida albicans

	C. glabrata
Gonorrhea	Neisseria gonorrhoeae
Toxoplasmosis	Toxoplasma gondii
Tetanus	Clostridium tetani
Caries	Streptococcus mutans
Whooping cough	Bordetella pertussis
Q fever endocarditis	Coxiella burnetti
Anthrax	Bacillus anthracis
Brucellosis	Brucella abortus

Numerous modifications and variations of the present invention are possible in light of the above teachings; therefore, within the scope of the appended claims the invention may be practiced otherwise than as particularly described.

WHAT IS CLAIMED IS:

1. A vaccine for protecting against an organism, comprising:

(a) a protein which is capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism, said native protein having at least 50% homology with a heat shock protein of T. cruzi; and

(b) a physiologically acceptable carrier.

2. A vaccine of claim 1 wherein the native protein is derived from a species of Mycoplasma, Mycobacteria, or Trypanosoma, provided that the native protein is not derived from Trypanosoma cruzi.

3. A vaccine of claim 2 wherein the native protein is derived from a species of Mycoplasma selected from the group consisting of M. mycoides, M. bovis, M. bovigenitalium, M. bovoculi, M. bovirhinis, M. dispar, M. hyorhinis, M. hyosynoviae, M. hyopneumoniae, M. gallisepticum, M. pneumoniae and M. synoviae.

4. A vaccine of claim 2 wherein the native protein is derived from a species of Mycobacteria selected from the group consisting of M. bovis, M. leprae and M. tuberculosis.

5. A vaccine of claim 3 wherein the native protein is derived from a species of Mycoplasma selected from the group consisting of M. hyopneumoniae and M. gallisepticum.

6. A process for protecting a host against an organism comprising:

administering an effective amount of a protein capable of eliciting an antibody which recognizes at least one epitope of a native protein present in the organism said native protein having at least 50% homology with a T. cruzi heat shock protein.

7. A process of claim 6 wherein the native protein is derived from a species of Mycoplasma, Mycobacteria, or Trypanosoma, provided that the native protein is not derived from Trypanosoma cruzi.

8. A process of claim 7 wherein the native protein is derived from a species of Mycoplasma selected from the group consisting of M. mycoides, M. bovis, M. bovigenitalium, M. bovoculi, M. bovirhinis, M. dispar, M. hyorhinis, M. hyosynoviae, M. hyopneumoniae, M. gallisepticum, M. pneumoniae, and M.. synoviae.

9. A process of claim 7 wherein the native protein is derived from a species of Mycobacteria selected from the group consisting of M. bovis, M. leprae, and M. tuberculosis.

10. A process of claim 8 wherein the native protein is derived from a species of Mycoplasma selected from the group consisting of M. hyopneumoniae and M. gallisepticum.

11. A process for determining an organism in a host comprising:

contacting a sample derived from a host containing or suspected of containing an organism with an antigen which is recognized by an antibody elicited in response to a protein present in the organism, said protein

having at least 50% homology with a heat shock protein of T. cruzi; and determining antibody in said sample bound by said antigen.

12. A process for determining an organism in a host, comprising:

contacting a sample derived from a host containing an organism or suspected of containing an organism with an antibody or fragment of said antibody, said antibody recognizing at least one epitope of a native protein present in the host, said native protein having at least 50% homology with a heat shock protein of T. cruzi; and

determining protein present in said organism bound to said antibody.

13. A process of claim 12 wherein the native protein is derived from a species of Mycoplasma, Mycobacteria, or Trypanosoma, provided that the native protein is not derived from Trypanosoma cruzi.

14. A process of claim 13 wherein the native protein is derived from a species of Mycoplasma selected from the group consisting of M. mycoides, M. bovis, M. bovigenitalium, M. bovoculi, M. bovirhinis, M. dispar, M. hyorhinis, M. hyosynoviae, M. hyopneumoniae, M. gallisepticum, M. pneumoniae and M. synoviae.

15. A process of claim 13 wherein the native protein is derived from a species of Mycobacteria selected from the group consisting of M. bovis, M. leprae, and M. tuberculosis.

16. A process of claim 14 wherein the native protein is derived from a species of Mycoplasma selected from the group consisting of M. hyopneumoniae and M. gallisepticum.

17. A recombinant sequence of nucleic acid encoding the heat shock proteins of M. hyopneumoniae and M. gallisepticum as depicted in figures 5 and 11, respectively.

FIGURE 1-1

Translation of clone pFP70-47

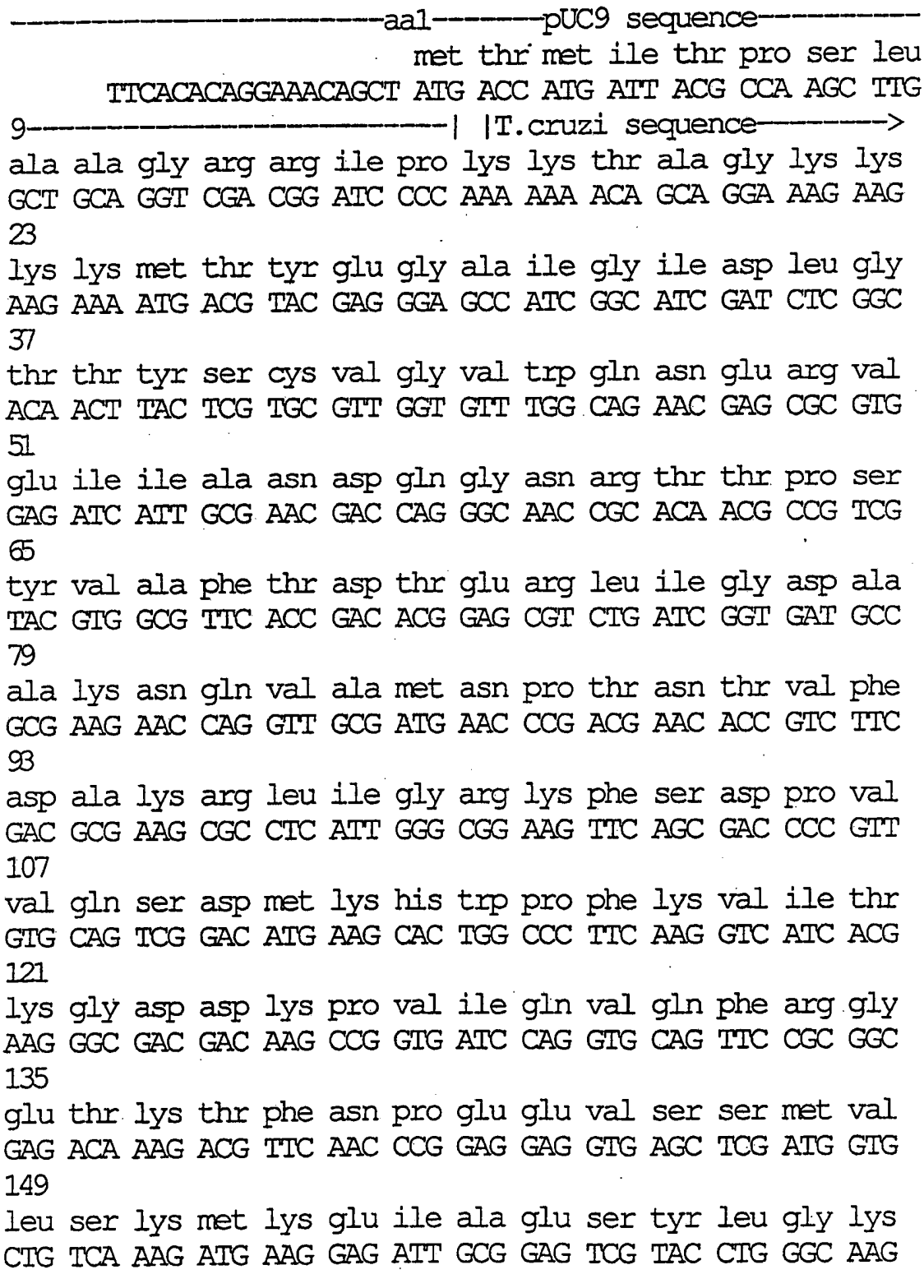


FIGURE 1-2

163
gln val lys lys ala val val thr val pro ala tyr phe asn
CAG GTG AAG AAG GCC GTG GTG ACT GTG CCC GCG TAC TTC AAC
187
asp ser gln arg gln ala thr lys asp ala gly thr ile ala
GAC TCC CAG CGG CAG GCG ACG AAG GAT GCC GGC ACG ATC GCG
201
gly met glu val leu arg ile ile asn glu pro thr ala ala
GGG ATG GAG GTG CTG CGC ATC ATC AAT GAG CCG ACA GCT GCC
215
ala ile ala tyr gly leu asp lys val glu asp gly lys glu
GCC ATT GCG TAC GGC CTG GAC AAA GTG GAG GAC GGC AAG GAG
239
arg asn val leu ile phe asp leu gly gly gly thr phe asp
CGC AAT GTG CTC ATC TTT GAC CTT GGC GGC GGC ACG TTT GAT
253
val thr leu leu thr ile asp gly gly ile phe glu val lys
GTC ACG CTG CTG ACG ATC GAC GGT GGC ATC TTT GAG GTG AAG
267
ala thr asn gly asp thr his leu gly gly glu asp phe asp
GCG ACG AAC GGC GAC ACG CAC CTG GGC GGC GAG GAC TTT GAC
281
asn arg leu val ser his phe thr asp glu phe lys arg lys
AAC CGC CTC GTG TCG CAC TTC ACG GAC GAG TTC AAG CGC AAG
295
asn lys gly lys asp leu thr thr ser gln arg ala leu arg
AAC AAG GGC AAG GAC CTG ACG ACA AGC CAG CGC GCC CTC CGC
309
arg leu arg thr ala cys glu arg ala lys arg thr leu ser
CGC CTC CGC ACC GCC TGC GAG CGC GCC AAG CGC ACG CTG TCG
323
ser ala ala gln ala thr ile glu ile asp ala leu phe asp
TCC GCG GCA CAG GCG ACG ATT GAG ATC GAC GCG CTG TTT GAC
337
asn val asp phe gln ala thr ile thr arg ala arg phe glu
AAC GTG GAC TTC CAG GCA ACC ATC ACT CGC GCC CGC TTC GAG

FIGURE 1-3

351
glu leu cys gly asp leu phe arg gly thr leu gln pro val
GAG CTC TGC GGC GAC CTC TTC CGA GGG ACG CTG CAG CCG GTG
365
glu arg val leu gln asp ala lys met asp lys arg ala val
GAG CGT GTG CTC CAG GAC GCC AAG ATG GAC AAG CGT GCC GTG
379
his asp val val leu val gly gly ser thr arg ile pro lys
CAC GAC GTG GTG CTC GTC GGC GGC TCC ACC CGC ATT CCA AAG
393
val met gln leu val ser asp phe phe gly gly lys glu leu
GTG ATG CAG CTG GTG TCT GAC TTT TTC GGT GGC AAG GAA CTG
407
asn lys ser ile asn pro asp glu ala val ala tyr gly ala
AAC AAG AGC ATC AAC CCT GAT GAG GCT GTG GCG TAC GGT GCC
421
ala val gln ala phe ile leu thr gly gly lys ser lys gln
GCC GTG CAG GCC TTC ATC CTG ACG GGC GGC AAG AGC AAG CAG
435
thr glu gly leu val leu leu asp val thr pro leu thr leu
ACG GAG GGC CTC GTG CTG CTC GAC GTG ACC CCG CTG ACG CTT
449
gly ile glu thr ala gly gly val met thr ser leu ile lys
GGC ATC GAG ACG GCG GGT GGC GTC ATG ACG TCG CTG ATC AAG
463
arg asn thr thr ile pro thr lys lys ser gln ile phe ser
CGC AAC ACG ACG ATT CCG ACC AAG AAA AGC CAG ATC TTC TCG
477
thr tyr ala asp asn gln pro gly val his ile gln val phe
ACG TAC GCG GAC AAC CAG CCG GGC GTG CAC ATC CAG GTC TTT
491
glu gly glu arg ala met thr lys asp cys his leu leu gly
GAG GGG GAG CGT GCG ATG ACG AAG GAC TGC CAC CTG CTC GGC
515
thr phe asp leu ser gly ile pro pro ala pro arg gly va
ACA TTC GAC CTG TCC GGC ATC CCG CCG GCG CCG CGC GGT GTG

FIGURE 1-4

529
pro gln ile glu val thr phe asp leu asp ala asn gly ile
CCC CAG ATT GAG GTT ACC TTT GAC CTC GAC GCC AAC GGC ATC
543
leu asn val ser ala glu glu lys gly thr gly lys arg asn
CTG AAC GTG TCC GCG GAG GAG AAG GGC ACC GGC AAG CGC AAC
557
gln ile val ile thr asn asp lys gly arg leu ser lys ala
CAG ATT GTC ATC ACG AAC GAC AAG GGC CGC CTG AGC AAG GCG
571
asp ile glu arg met val ser glu ala ala lys tyr glu ser
GAC ATT GAG CGC ATG GTG TCC GAG GCT GCC AAG TAC GAG TCG
585
gln asp lys glu gln arg glu arg ile asp ala lys asn gly
CAG GAC AAG GAA CAG CGC GAG CGC ATT GAC GCA AAG AAC GGT
599
leu glu asn tyr ala phe ser val lys asn thr val asn glu
CTT GAG AAC TAC GCA TTT TCG GTG AAG AAC ACC GTA AAC GAG
613
pro asn val ala gly lys ile glu glu ala asp lys asn thr
CCG AAC GTC GCT GGC AAG ATT GAG GAG GCC GAC AAG AAC ACG
627
ile thr ser ala val glu glu ala leu gln trp leu asn asn
ATT ACG AGT GCC GTG GAG GAG GCG CTG CAA TGG CTG AAC AAC

FIGURE 1-5

641
asn gln glu ala ser lys glu glu tyr glu his arg gln lys
AAC CAG GAG GCC AGC AAG GAG GAG TAC GAG CAC CGC CAG AAG
655
glu leu glu asn leu cys thr pro ile met thr lys met tyr
GAG CTG GAG AAC CTG TGC ACG CCC ATC ATG ACG AAG ATG TAC
669
gln gly met gly ala gly gly gly met pro gly gly met pro
CAG GGC ATG GGC GCG GGC GGC GGT ATG CCC GGA GGT ATG CCT
683
gly gly met pro gly gly met pro gly gly ala asn pro ser
GGT GGA ATG CCC GGG GGC ATG CCT GGT GGC GCG AAC CCG TCG
697
ser ser ser gly pro lys val glu glu val asp OP
TCT TCG TCA GGA CCG AAG GTG GAG GAA GTG GAC TGA GAGCGCATCC

CTGAAGATGTTCCCATGGCGGCGTCTGCTCGCGAACGAATAACCCGTTGGTTTTCTCC

CTTGTAGAGCGTAGAGGTCTGCGACAAACCCAGCCGCCATCACTATTTTTATTATTGG

TTTTTTTCCCTCTCCATTATTATTATTATTATTATTATTATTACGGTTGTTATTT

GTATTGTCATTGCGATGGCACTTGTGCTGTTGAGGGCACCACGGTTGCCTCTGCCATT

TTTGTTGCTGACTGACGCCTGTGTGCGTCTCCTTGTACCGCCGGCTTCCTTTCCTCCT

TTCTCCCCCGCTCCTTCGCCCTGT

FIGURE 2-1

CLUSTERED PAIR-WISE ALIGNMENT listed in clustered order, in 'identity (no translation)' alphabet of:

1.	Mhyhsp70	(1-600)	7.	x170	(1-647)
2.	Bmehsp70	(1-605)	8.	humhsp70	(1-641)
3.	dnaK	(1-638)	9.	chkhsp70	(1-635)
4.	tc70kd	(1-669)	10.	mzehsp70	(1-646)
6.	rathsp70	(1-646)	11.	smahsp70	(1-620)

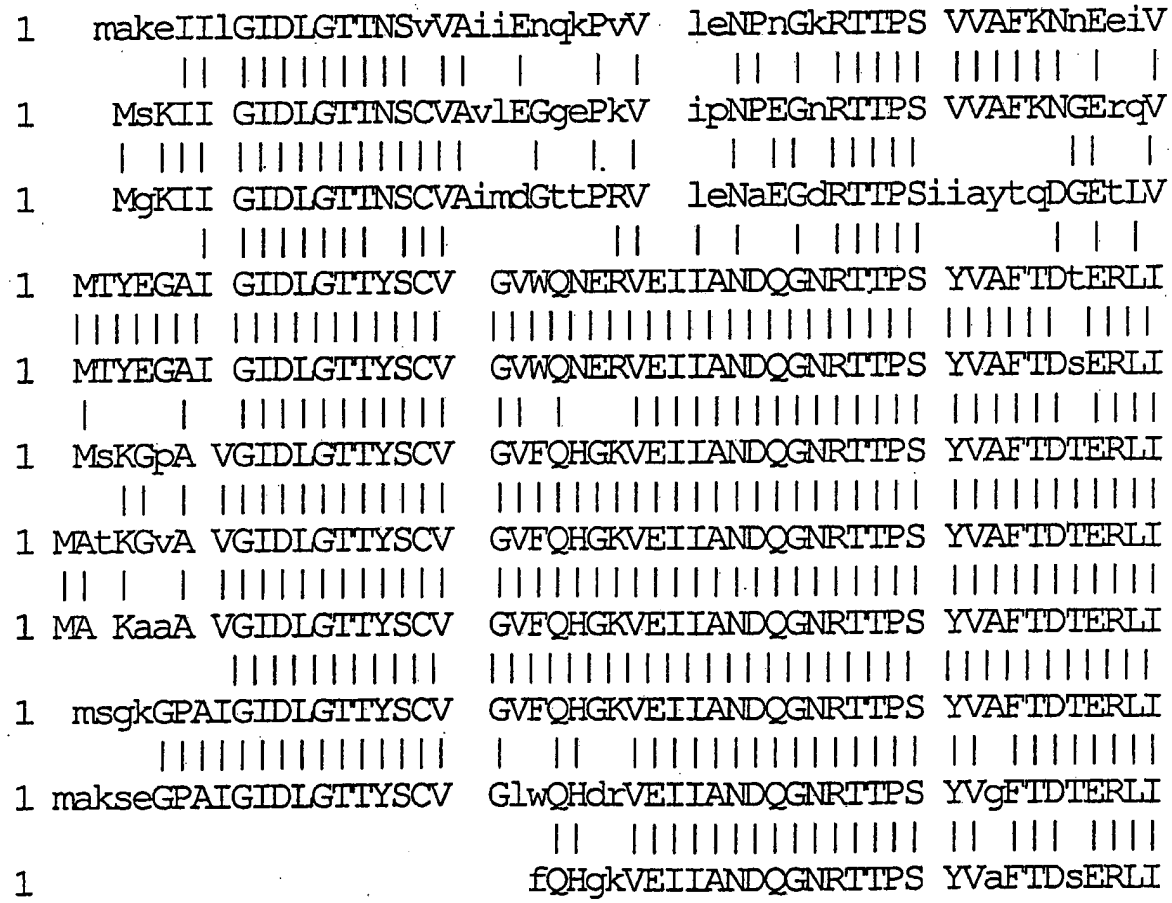


FIGURE 2-2

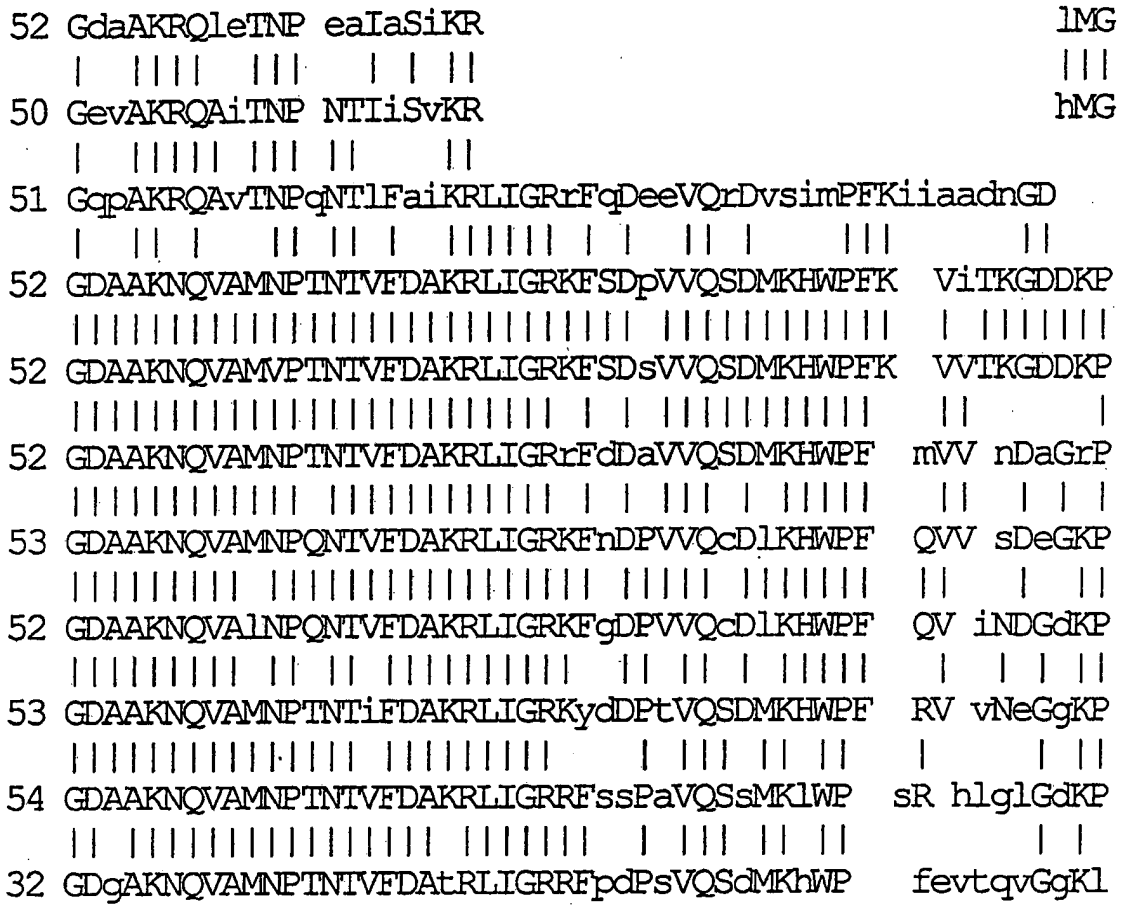

52 GdaAKRQleTNP eaIaSiKR lMG
50 GevAKRQAiTNP NTIiSvKR hMG
51 GqpAKRQAvTNPqNTlFaiKRLIGRrFqDeeVQrDvsimPFKiiaadnGD
52 GDAAKNQVAMNPTNIVFDAKRLIGRKFSDpVVQSDMKHWPFK ViTKGDDKP
52 GDAAKNQVAMVPTNIVFDAKRLIGRKFSDsVVQSDMKHWPFK VVIKGDDKP
52 GDAAKNQVAMNPTNIVFDAKRLIGRrFdDaVVQSDMKHWPF mVV nDaGrP
53 GDAAKNQVAMNPQNIVFDAKRLIGRKFnDPVVQcDlKHWPF QVV sDeGKP
52 GDAAKNQVAlNPQNIVFDAKRLIGRKFgDPVVQcDlKHWPF QV iNDGdKP
53 GDAAKNQVAMNPTNTiFDAKRLIGRKydDPtVQSDMKHWPF RV vNeGgKP
54 GDAAKNQVAMNPTNIVFDAKRLIGRRFssPaVQSsMKlWP sR hlglGdKP
32 GDgAKNQVAMNPTNIVFDAtRLIGRRFpdPsVQSdMKhWP fevtqvGgKl

FIGURE 2-3

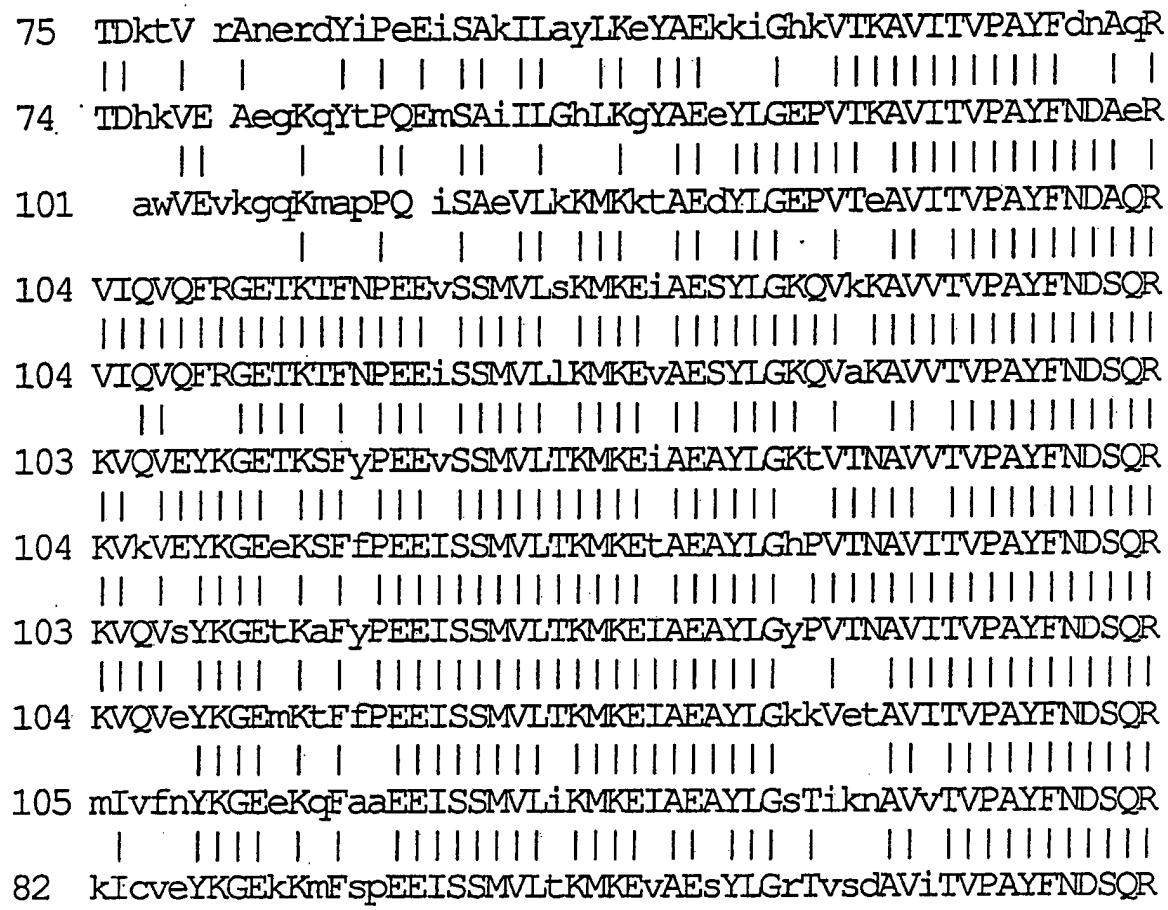

75 TDktV rAnerdYiPeEiSAkILayLKeYAEkkiGhkVTKAVITVPAYFdnAqR
74 TDhkVE AegKqYtPQEmSAiILGhLKgYAEeYLGEPVTKAVITVPAYFNDAeR
101 awVEvkgqKmapPQ iSAeVLkKMKktAEdYLGEPVTeAVITVPAYFNDAQR
104 VIQVQFRGETKTFNPEEvSSMVLsKMKEiAESYLGKQVkKAVVTVPAYFNDSQR
104 VIQVQFRGETKTFNPEEiSSMVLlKMKEvAESYLGKQVaKAVVTVPAYFNDSQR
103 KVQVEYKGETKSFyPEEvSSMVLTKMKEiAEAYLGKtVTNAVVTVPAYFNDSQR
104 KVkVEYKGEeKSFfPEEISSMVLTKMKEtAEAYLGhPVTNAVITVPAYFNDSQR
103 KVQVsYKGEtKaFyPEEISSMVLTKMKEIAEAYLGyPVTNAVITVPAYFNDSQR
104 KVQVeYKGEmKtFfPEEISSMVLTKMKEIAEAYLGkkVetAVITVPAYFNDSQR
105 mIvfnYKGEeKqFaaEEISSMVLiKMKEIAEAYLGsTiknAVvTVPAYFNDSQR
82 kIcveYKGEkKmFspEEISSMVLtKMKEvAEsYLGrIvsdAViTVPAYFNDSQR

FIGURE 2-4

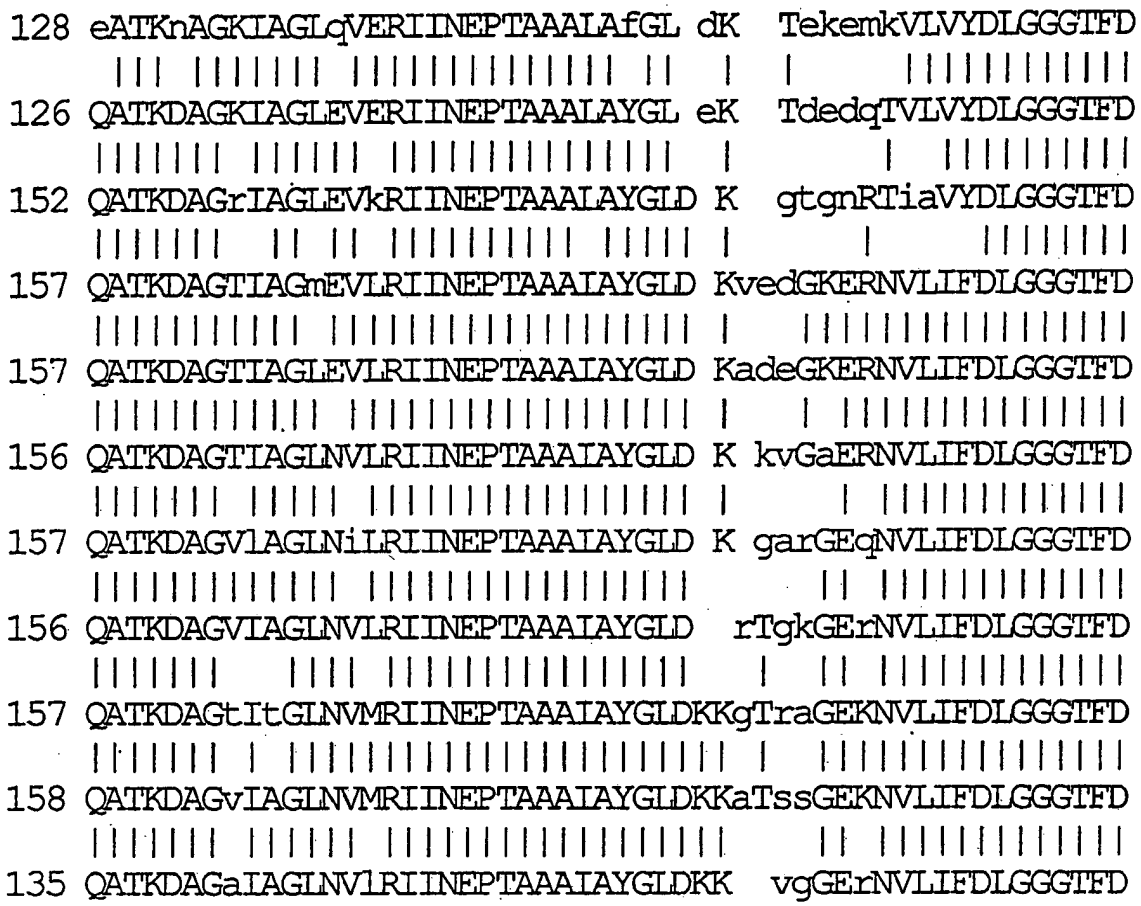

128 eATKnAGKIAGLqVERIINEPTAAALAfGL dK TekemkVLVYDLGGGTFD
126 QATKDAGKIAGLEVERIINEPTAAALAYGL eK TdedqIVLVYDLGGGTFD
152 QATKDAGrIAGLEVkRIINEPTAAALAYGLD K gtgnRTiaVYDLGGGTFD
157 QATKDAGTIAGmEVLRIINEPTAAAIAYGLD KvedGKERNVLIFDLGGGTFD
157 QATKDAGTIAGLEVLRIINEPTAAAIAYGLD KadeGKERNVLIFDLGGGTFD
156 QATKDAGTIAGLNVLRIINEPTAAAIAYGLD K kvGaERNVLIFDLGGGTFD
157 QATKDAGVlAGLNiLRIINEPTAAAIAYGLD K garGEqNVLIFDLGGGTFD
156 QATKDAGVIAGLNVLRIINEPTAAAIAYGLD rTgkGErNVLIFDLGGGTFD
157 QATKDAGtItGLNVMRIINEPTAAAIAYGLDKKgTraGEKNVLIFDLGGGTFD
158 QATKDAGvIAGLNVMRIINEPTAAAIAYGLDKKaTssGEKNVLIFDLGGGTFD
135 QATKDAGaIAGLNVlRIINEPTAAAIAYGLDKK vgGErNVLIFDLGGGTFD

FIGURE 2-5

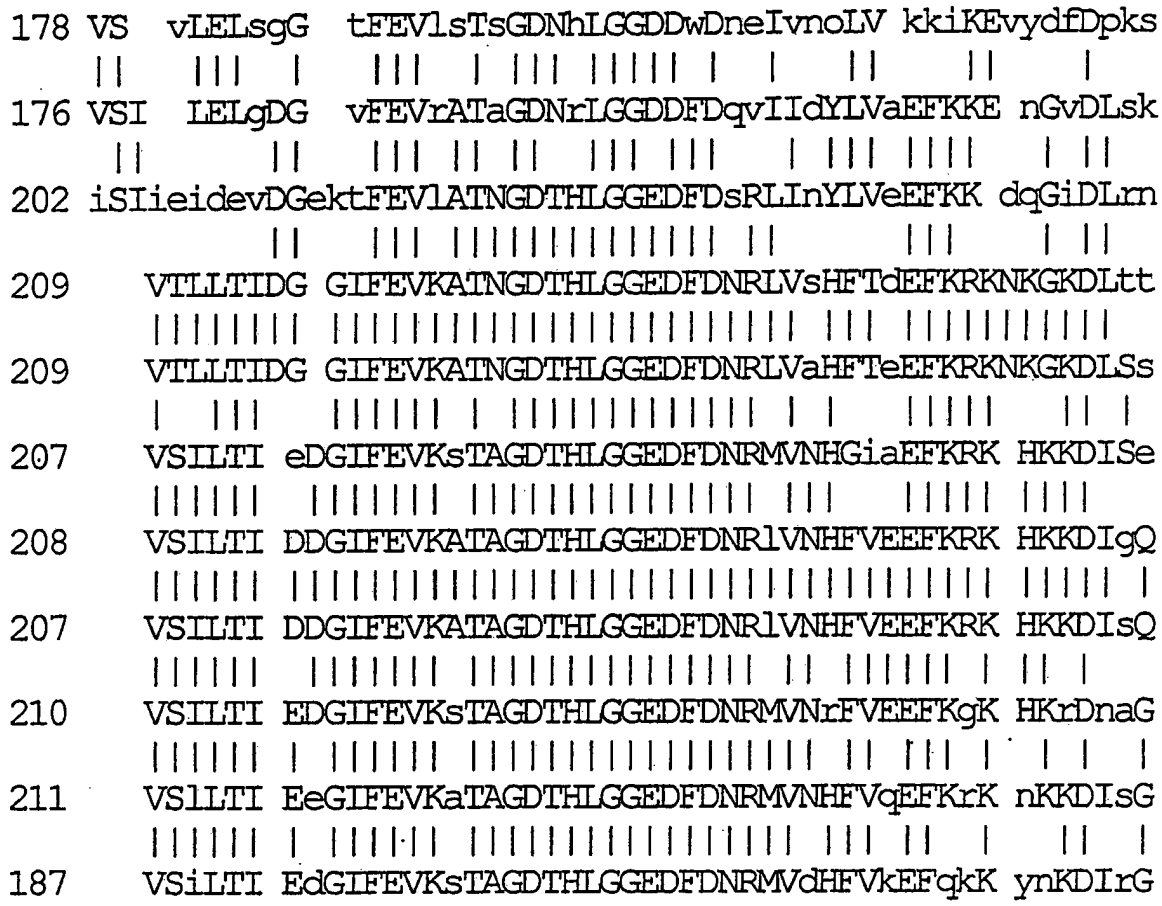

178 VS vLELsgG tFEVlsTsGDNhLGGDDwDneIvnoLV kkiKEvydfDpks
176 VSI LELgDG vFEVrATaGDNrLGGDDFDqvIIdYLVaEFKKE nGvDLsk
202 iSIieidevDGektFEVlATNGDTHLGGEDFDsRLInYLVeEFKK dqGiDLrn
209 VTLLTIDG GIFEVKATNGDTHLGGEDFDNRLVsHFTdEFKRKNKGKDLtt
209 VTLLTIDG GIFEVKATNGDTHLGGEDFDNRLVaHFTeEFKRKNKGKDLSs
207 VSILTI eDGIFEVKsTAGDTHLGGEDFDNRMVNHGiaEFKRK HKKDISe
208 VSILTI DDGIFEVKATAGDTHLGGEDFDNRlVNHFVEEFKRK HKKDIgQ
207 VSILTI DDGIFEVKATAGDTHLGGEDFDNRlVNHFVEEFKRK HKKDIsQ
210 VSILTI EDGIFEVKsTAGDTHLGGEDFDNRMVNrFVEEFKgK HKrDnaG
211 VSlLTI EeGIFEVKaTAGDTHLGGEDFDNRMVNHFVqEFKrK nKKDIsG
187 VSiLTI EdGIFEVKsTAGDTHLGGEDFDNRMVdHFVkEFqkK ynKDIrG

FIGURE 2-6


227 DKMALtRLKeeAEKtKinLSnqsvST vSLPFlgmGknGPinveLE LkRseF
225 DKMALQRLKdAAEKAKkdLS gvtSTqiSLPFITAGeaGP lHLEvsLsRAKF
255 DplAmQRLKeAAEKAKieLS SAqqTdvnLPyITADAtGP kHmnikvTRAKl
259 sqRALRRLRTACERAKRTLS SA AQATIEIDA LFdNvDFQATITRARF
259 NlRALRRLRTACERAKRTLS SA AQATIEIDA LFENIDFQATITRARF
256 NKRAvRRLRTACERAKRTLS S StQASIEID SLyEGIDFYTsITRARF
257 NKRAlRRLRTACdRAKRTLS S SsQASIEID SLFEGIDFYTaITRARF
256 NKRAVRRLRTACERAKRTLS S STgASlEID SLFEGIDFYTSITRARF
257 NKRAVRRLRTACERArRTLS S STQASIEID SLFEGIDFYTSITRARF
258 NpRALRRLRTACERAKRTLS S tAQTtIEID SLFEGIDFtprssRARF
236 NkRALRRLRTACERAKRTLS S sAQInlEID SLodGIDFytvitRARF

FIGURE 2-7

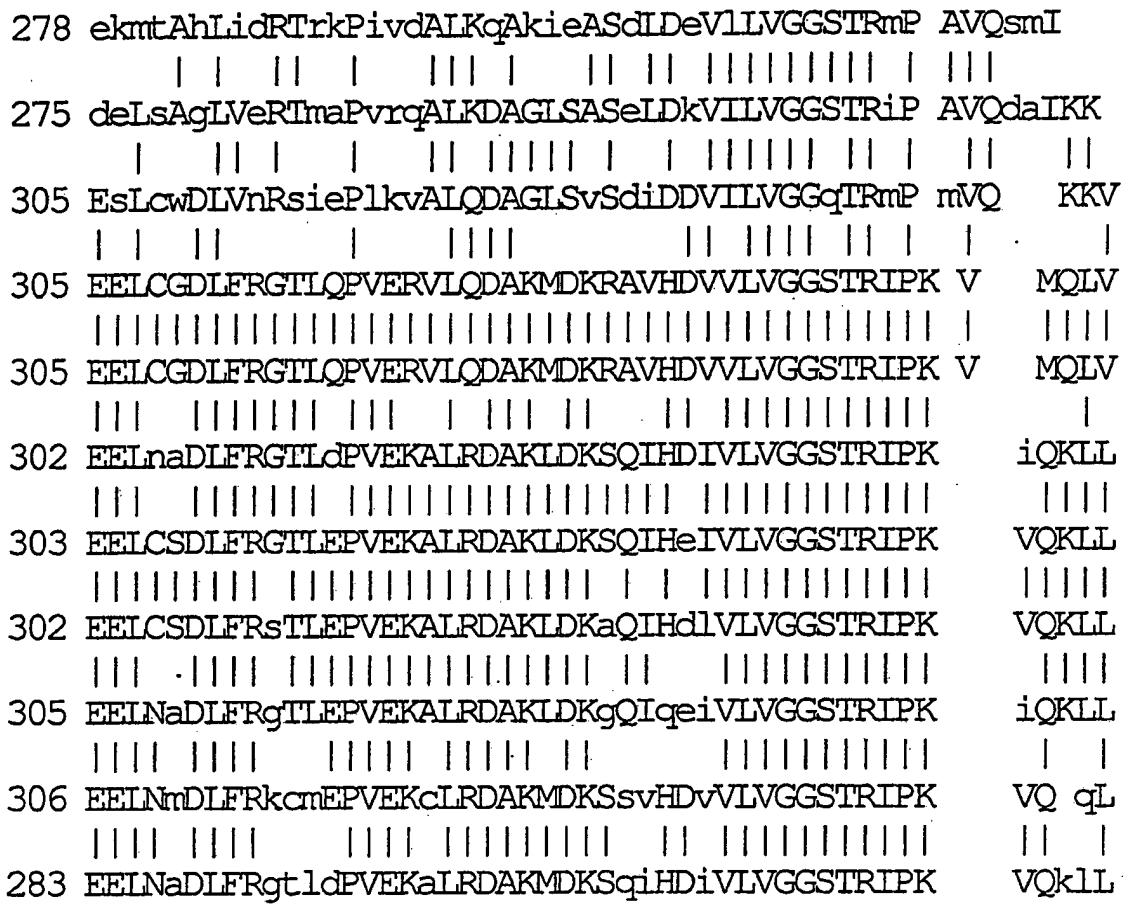

278 ekmtAhLidRTrkPivdALKqAkieASdLDeVlLVGGSTRmP AVQsmI
275 deLsAgLVeRImaPvrqALKDAGLSASeLDkVILVGGSTRiP AVQdaIKK
305 EsLcwDLVnRsiePlkvALQDAGLSvSdiDDVILVGGqTRmP mVQ KKV
305 EELCGDLFRGTLQPVERVLQDAKMDKRAVHDVVLVGGSTRIPK V MQLV
305 EELCGDLFRGTLQPVERVLQDAKMDKRAVHDVVLVGGSTRIPK V MQLV
302 EELnaDLFRGTLdPVEKALRDAKLDKSQIHDIVLVGGSTRIPK iQKLL
303 EELCSDLFRGTLEPVEKALRDAKLDKSQIHeIVLVGGSTRIPK VQKLL
302 EELCSDLFRsTLEPVEKALRDAKLDKaQIHdlVLVGGSTRIPK VQKLL
305 EELNaDLFRgTLEPVEKALRDAKLDKgQIqeiVLVGGSTRIPK iQKLL
306 EELNmDLFRkcmEPVEKcLRDAKMDKSsvHDvVLVGGSTRIPK VQ qL
283 EELNaDLFRgtldPVEKaLRDAKMDKSqiHDiVLVGGSTRIPK VQklL

FIGURE 2-8

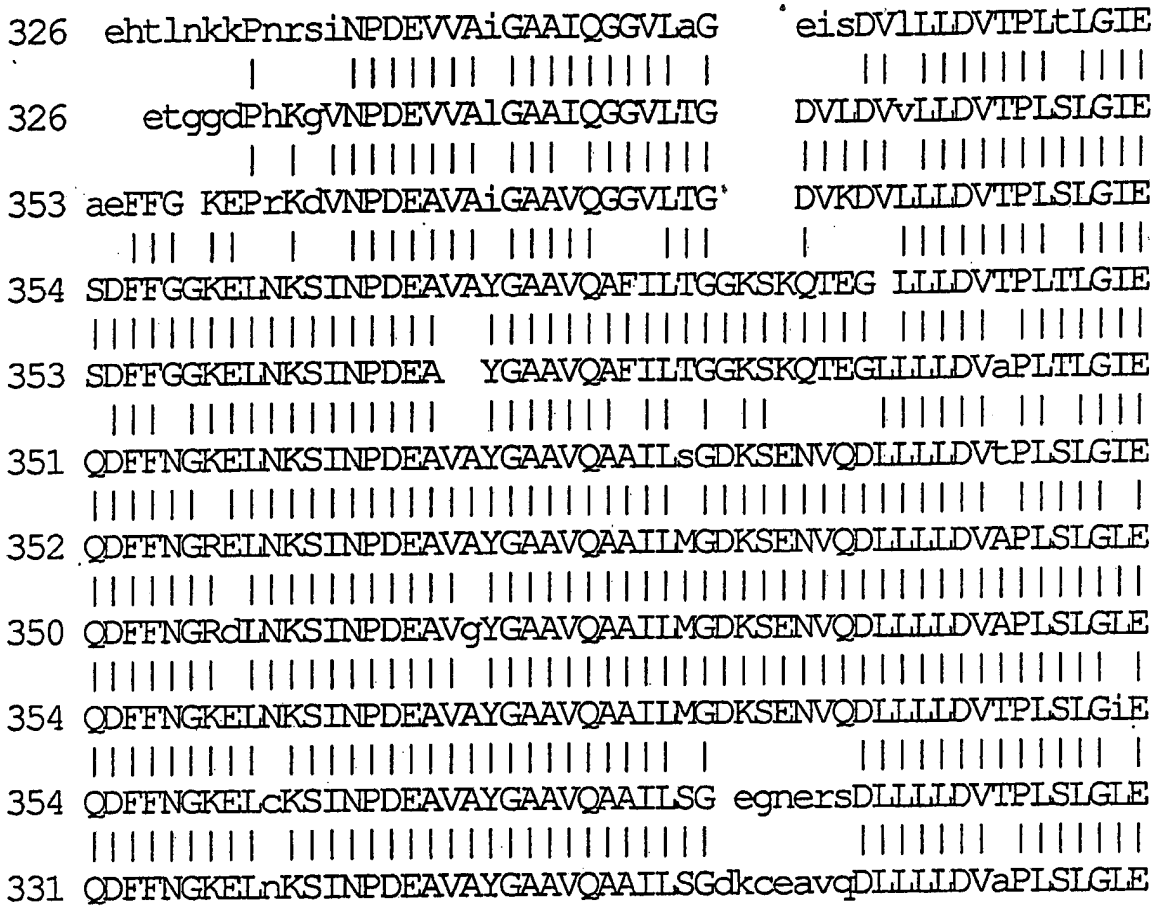

326 ehtlnkkPnrsiNPDEVVAiGAAIQGGVLaG eisDVlLLDVTPLtLGIE
326 etggdPhKgVNPDEVVAlGAAIQGGVLTG DVLDVvLLDVTPLSLGIE
353 aeFFG KEPrKdVNPDEAVAiGAAVQGGVLTG DVKDVLLLDVTPLSLGIE
354 SDFFGGKELNKSINPDEAVAYGAAVQAFILTGGKSKQTEG LLLDVTPLTLGIE
353 SDFFGGKELNKSINPDEA YGAAVQAFILTGGKSKQTEGLLLLDVaPLTLGIE
351 QDFFNGKELNKSINPDEAVAYGAAVQAAILsGDKSENVQDLLLLDVtPLSLGIE
352 QDFFNGRELNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLE
350 QDFFNGRdLNKSINPDEAVgYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLE
354 QDFFNGKELNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVTPLSLGiE
354 QDFFNGKELcKSINPDEAVAYGAAVQAAILSG egnersDLLLLDVTPLSLGLE
331 QDFFNGKELnKSINPDEAVAYGAAVQAAILSGdkceavqDLLLLDVaPLSLGLE

FIGURE 2-9

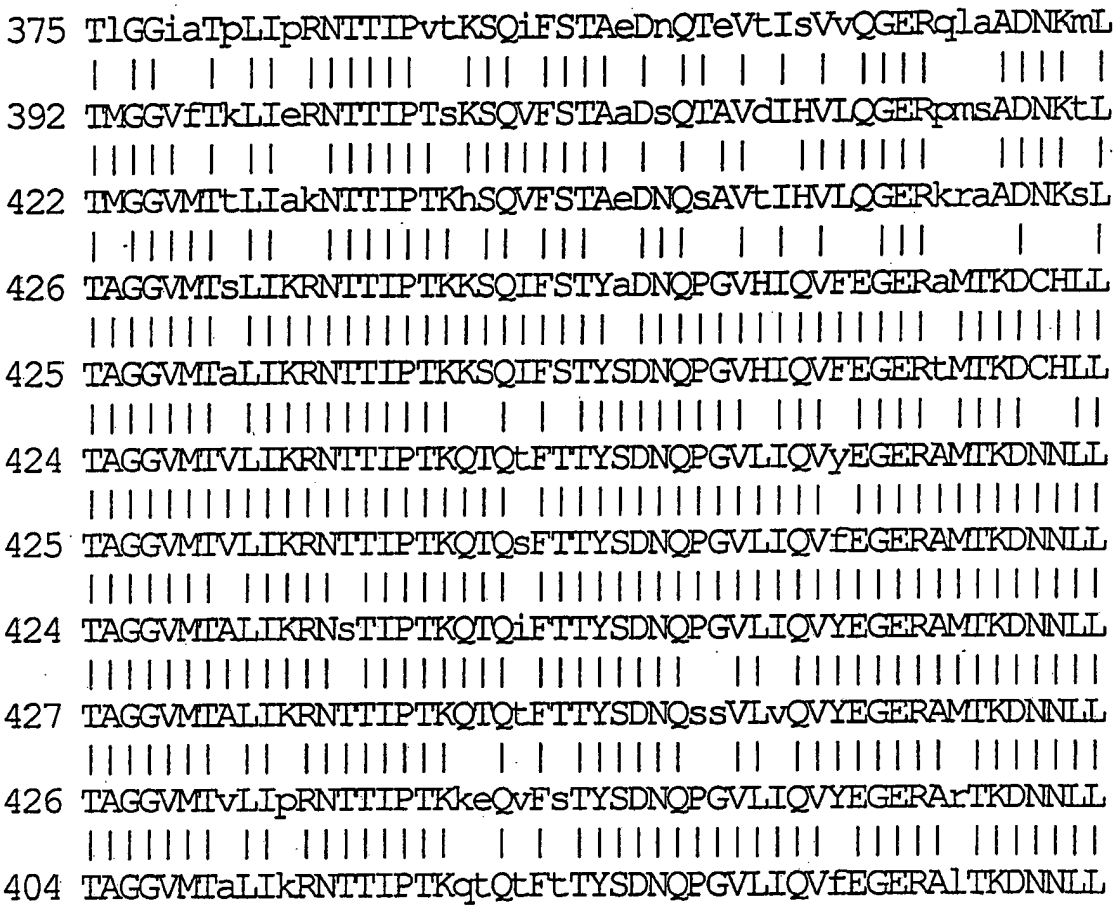

375 TlGGiaTpLIpRNTTIPvtKSQiFSTAeDnQTeVtIsVvQGERqlaADNKmL
392 TMGGVfTkLIeRNTTIPTsKSQVFSTAaDsQTAVdIHVLQGERpmsADNKtL
422 TMGGVMTtLIakNTTIPTKhSQVFSTAeDNQsAVtIHVLQGERkraADNKsL
426 TAGGVMTsLIKRNTTIPTKKSQIFSTYaDNQPGVHIQVFEGERaMTKDCHLL
425 TAGGVMTaLIKRNTTIPTKKSQIFSTYSDNQPGVHIQVFEGERtMTKDCHLL
424 TAGGVMTVLIKRNTTIPTKQTQtFTTYSDNQPGVLIQVyEGERAMTKDNNLL
425 TAGGVMTVLIKRNTTIPTKQTQsFTTYSDNQPGVLIQVfEGERAMTKDNNLL
424 TAGGVMTALIKRNsTIPTKQTQiFTTYSDNQPGVLIQVYEGERAMTKDNNLL
427 TAGGVMTALIKRNTTIPTKQTQtFTTYSDNQssVLvQVYEGERAMTKDNNLL
426 TAGGVMTvLIpRNTTIPTKkeQvFsTYSDNQPGVLIQVYEGERArTKDNNLL
404 TAGGVMTaLIkRNTTIPTKqtQtFtTYSDNQPGVLIQVfEGERAlTKDNNLL

FIGURE 2-10

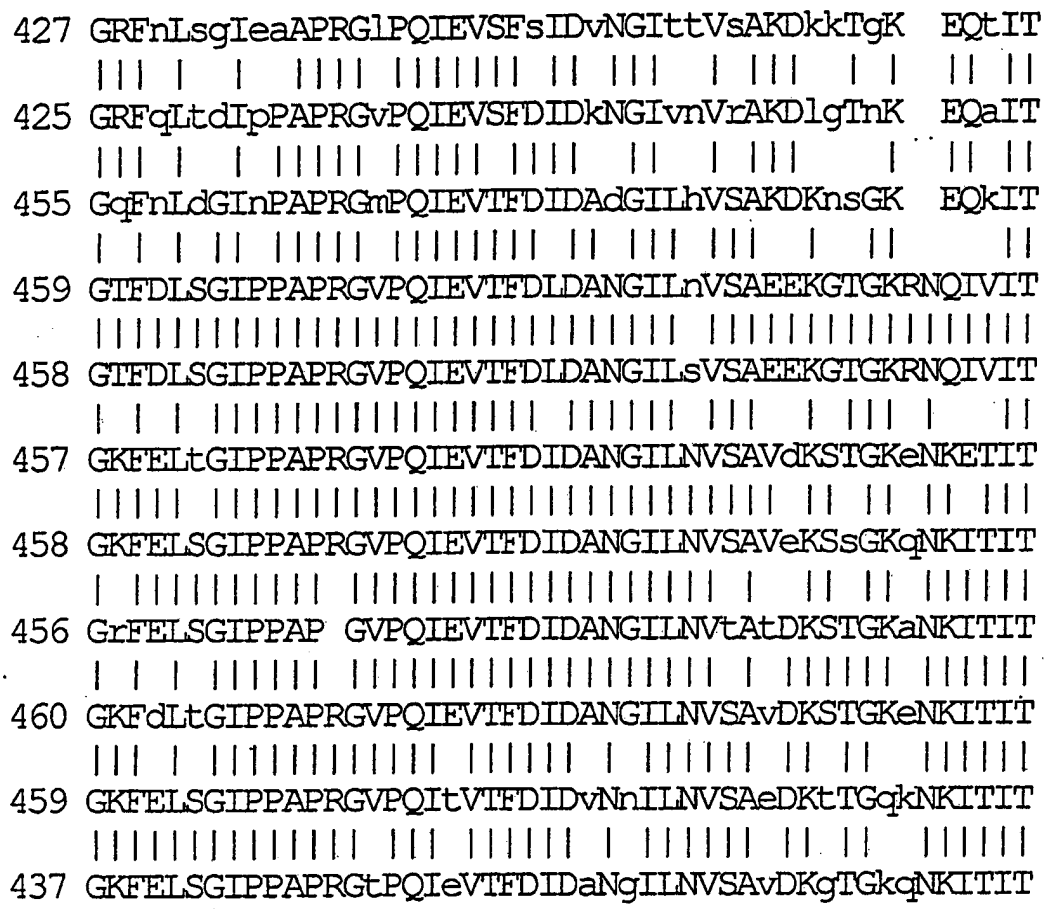

427 GRFnLsgIeaAPRGlPQIEVSFsIDvNGIttVsAKDkkTgK EQtIT
425 GRFqLtdIpPAPRGvPQIEVSFDIDkNGIvnVrAKDlgTnK EQaIT
455 GqFnLdGInPAPRGmPQIEVTFDIDAdGILhVSAKDKnsGK EQkIT
459 GTFDLSGIPPAPRGVPQIEVTFDLDANGILnVSAEEKGTGKRNQIVIT
458 GTFDLSGIPPAPRGVPQIEVTFDLDANGILsVSAEEKGTGKRNQIVIT
457 GKFELtGIPPAPRGVPQIEVTFDIDANGILNVSAVdKSTGKeNKETIT
458 GKFELSGIPPAPRGVPQIEVTFDIDANGILNVSAVeKSsGKqNKITIT
456 GrFELSGIPPAP GVPQIEVTFDIDANGILNVtAtDKSTGKaNKITIT
460 GKFdLtGIPPAPRGVPQIEVTFDIDANGILNVSAvDKSTGKeNKITIT
459 GKFELSGIPPAPRGVPQItVTFDIDvNnILNVSAeDKtTGqkNKITIT
437 GKFELSGIPPAPRGtPQIeVTFDIDaNgILNVSAvDKgTGkqNKITIT

FIGURE 2-11

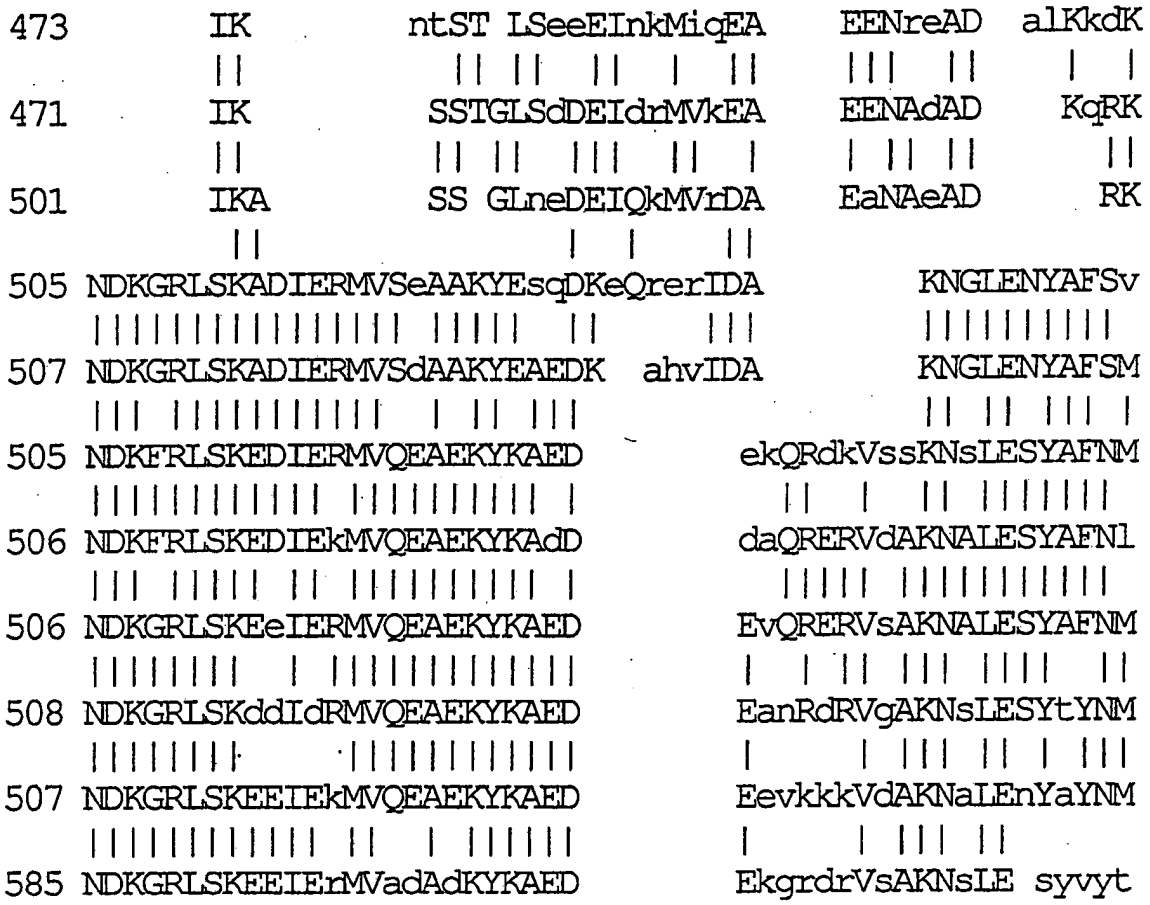

473 IK ntST LSeeEInkMiqEA EENreAD alKkdK
471 IK SSTGLSdDEIdrMVkEA EENAdAD KqRK
501 IKA SS GLneDEIQkMVrDA EaNAeAD RK
505 NDKGRLSKADIERMVSeAAKYEsqDKeQrerIDA KNGLENYAFSv
507 NDKGRLSKADIERMVSdAAKYEAEDK ahvIDA KNGLENYAFSM
505 NDKFRLSKEDIERMVQEAEKYKAED ekQRdkVssKNsLESYAFNM
506 NDKFRLSKEDIEkMVQEAEKYKAdD daQRERVdAKNALESYAFNl
506 NDKGRLSKEeIERMVQEAEKYKAED EvQRERVsAKNALESYAFNM
508 NDKGRLSKddIdRMVQEAEKYKAED EanRdRVgAKNsLESYtYNM
507 NDKGRLSKEEIEkMVQEAEKYKAED EevkkkVdAKNaLEnYaYNM
585 NDKGRLSKEEIErMVadAdKYKAED EkgrdrVsAKNsLE syvyt

FIGURE 2-12


505 iEttVraeglinQL EKsitDqgeK idpkqkellekq
501 EE VelRNeadQLvfttEKtLkDlegKVEEA evtkanea
530 fEElVqtRN qgdhlLhstrkqVEEA gdklpaddKtaiEsaltaL
552 KNTvNePNVAGK ieeADKNtiTsAVEEALqWLNnNQEASKEEYEHRQKEL
549 KNTiNDPNVAGK ldDADKNavTtAVEEALrWLNdNQEASlEEYnHRQKEL
550 KaTVEDEklqGKI nDEDKqkIldKCnEiISWLdkNQtAEKEEfeHQQKEL
551 KSmVEDEnvKGKI SDEDKrtIseKCtqVISWLenNQLAEKEEyafQQKdL
552 KSaVEDEgLKGKI SeaDKkKVLDKCQEVISWLDaNtLAEKdEfEHKrKEL
556 KqtVEDEkLKGKI SdqDKqKVLDKCQEVISsLDrNQmAEKEEyEHKqKEL
555 rntikddKIasklpaeDKkKiEDavdgaISWLDsNQlAEvEEfEdKmKEL
529 mkqqvegelkeKIpesdhqviisKcED tISWLDvhQsAEkhEyesKreEL

FIGURE 2-13

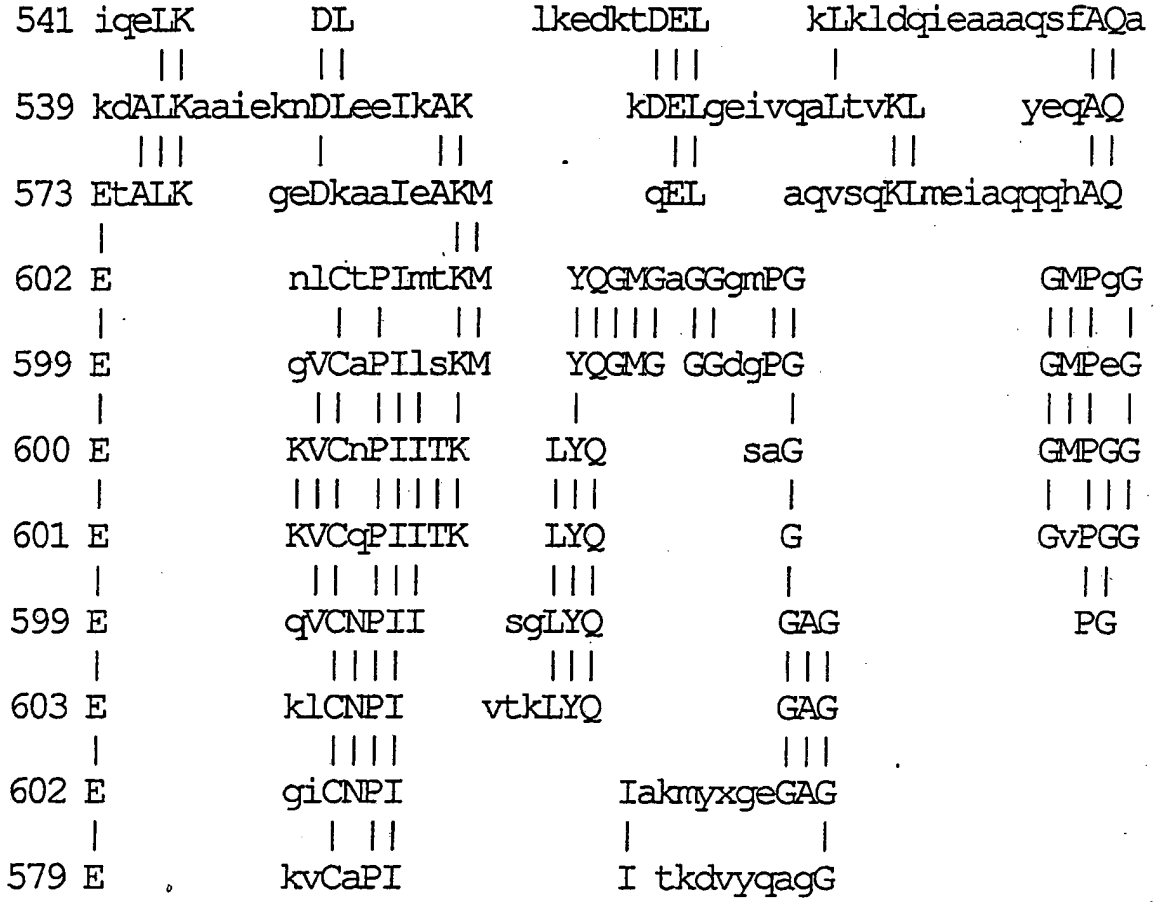

541 iqeLK DL lkedktDEL kLkldqieaaaqsfAQa
539 kdALKaaieknDLeeIkAK kDELgeivqaLtvKL yeqAQ
573 EtALK geDkaaIeAKM qEL aqvsqKLmeiaqqqhAQ
602 E nlCtPImtKM YQGMGaGGgmPG GMPgG
599 E gVCaPIlsKM YQGMG GGdgPG GMPeG
600 E KVCnPIITK LYQ saG GMPGG
601 E KVCqPIITK LYQ G GvPGG
599 E qVCNPII sgLYQ GAG PG
603 E klCNPI vtkLYQ GAG
602 E giCNPI Iakmyxge GAG
579 E kvCaPI I tkdvyqagG

FIGURE 2-14

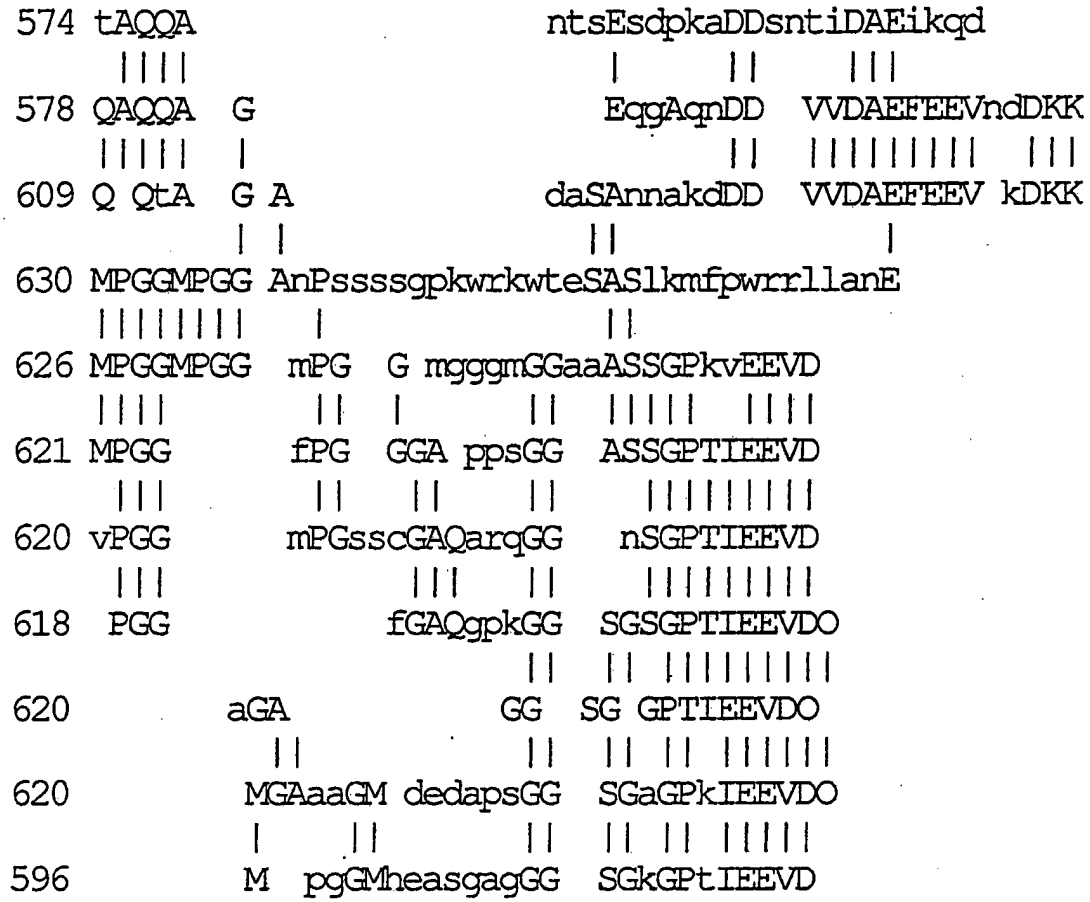
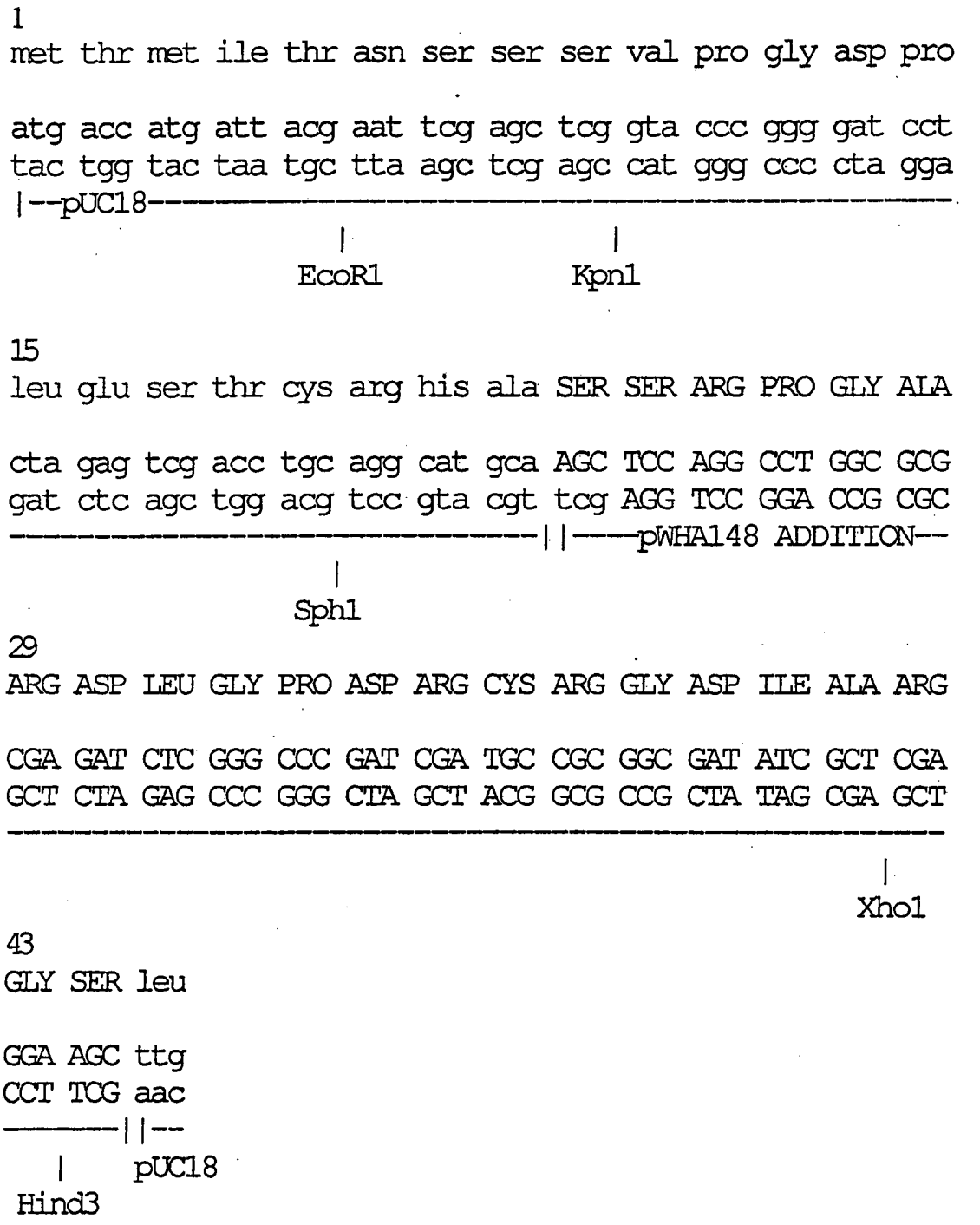

574 tAQQA ntsEsdpkaDDsntiDAEikqd
578 QAQQA G EqgAqnDD VVDAEFEEVndDKK
609 Q QtA G A daSAnnakdDD VVDAEFEEV kDKK
630 MPGGMPGG AnPssssgpkwrkwteSASlkmfpwrrllanE
626 MPGGMPGG mPG G mgggmGGaaASSGPkvEEVD
621 MPGG fPG GGA ppsGG ASSGPTIEEVD
620 vPGG mPGsscGAQarqGG nSGPTIEEVD
618 PGG fGAQgpkGG SGSGPTIEEVDO
620 aGA GG SG GPTIEEVDO
620 MGAaaGM dedapsGG SGaGPkIEEVDO
596 M pgGMheasgagGG SGkGPtIEEVD

FIGURE 4-1



Position of pUC18 conserved sequences, addition endpoints and predicted partial amino acid sequence of the beta-galactosidase fusion protein produced in pWHA148. A portion of the nucleotide sequence of pUC18 is designated

FIGURE 4-2

by lower case letters; the nucleotide sequence of the pWHA148 synthetic oligonucleotide addition is designated by upper case letters. Numbers refer to the order of the espected amino acid sequence.

FIGURE 5-1

Translation of M. hyopneumoniae 74.5kD Antigen Gene

1
met ala lys glu ile ile leu gly ile asp leu gly thr thr
ATG GCA AAA GAA ATC ATT TTA GGA ATC GAC CTT GGA ACA ACA
15
asn ser val val ala ile ile glu asn gln lys pro val val
AAC TCA GTT GTT GCA ATT ATT GAA AAT CAA AAA CCT GTC GTT
30
leu glu asn pro asn gly lys arg thr thr pro ser val val
CTC GAA AAT CCC AAC GGA AAA AGA ACA ACT CCA TCC GTT GTC
45
ala phe lys asn asn glu glu ile val gly asp ala ala lys
GCT TTT AAA AAC AAT GAA GAA ATT GTC GGG GAT GCA GCT AAA
60
arg gln leu glu thr asn pro glu ala ile ala ser ile lys
AGA CAA CTT GAA ACT AAC CCA GAA GCA ATC GCT TCA ATT AAA
75
arg leu met gly thr asp lys thr val arg ala asn glu arg
AGA TTA ATG GGA ACT GAT AAA ACA GTT CGT GCA AAT GAA AGA
90
asp tyr ile pro glu glu ile ser ala lys ile leu ala tyr
GAT TAT ATT CCT GAA GAA ATC TCG GCA AAA ATT CTT GCT TAT
105
leu lys glu tyr ala glu lys lys ile gly his lys val thr
TTA AAA GAA TAT GCT GAG AAA AAG ATT GGT CAT AAA GTA ACA
120
lys ala val ile thr val pro ala tyr phe asp asn ala gln
AAA GCA GTA ATT ACA GTA CCT GCT TAT TTT GAC AAT GCC CAA
135
arg glu ala thr lys asn ala gly lys ile ala gly leu gln
CGT GAG GCA ACA AAA AAT GCC GGA AAA ATC GCT GGA TTA CAA
150
val glu arg ile ile asn glu pro thr ala ala ala leu ala
GTA GAA AGA ATT ATA AAT GAA CCA ACA GCG GCC GCA CTT GCT
165
phe gly leu asp lys thr glu lys glu met lys val leu val
TTT GGC CTT GAT AAA ACT GAA AAA GAA ATG AAA GTT CTT GTC

FIGURE 5-2

180
tyr asp leu gly gly gly thr phe asp val ser val leu glu
TAT GAC TTA GGT GGG GGA ACT TTT GAT GTC TCA GTT TTA GAA
195
leu ser gly gly thr phe glu val leu ser thr ser gly asp
TTA TCC GGT GGA ACC TTC GAA GTT TTA TCA ACA AGT GGT GAT
210
asn his leu gly gly asp asp trp asp asn glu ile val asn
AAT CAT TTA GGT GGG GAT GAC TGG GAT AAT GAA ATT GTA AAT
225
trp leu val lys lys ile lys glu val tyr asp phe asp pro
TGA CTT GTT AAA AAA ATC AAA GAA GTA TAT GAT TTT GAT CCA
240
lys ser asp lys met ala leu thr arg leu lys glu glu ala
AAA AGT GAT AAA ATG GCG CTT ACA AGA CTT AAA GAA GAG GCT
255
glu lys thr lys ile asn leu ser asn gln ser val ser thr
GAA AAA ACC AAA ATT AAT CTT TCA AAT CAA AGT GTT TCT ACA
270
val ser leu pro phe leu gly met gly lys asn gly pro ile
GTT TCT CTA CCA TTT TTA GGA ATG GGC AAA AAC GGG CCG ATT
285
asn val glu leu glu leu lys arg ser glu phe glu lys met
AAC GTT GAA CTT GAA CTT AAA AGA TCA GAA TTT GAA AAA ATG
300
thr ala his leu ile asp arg thr arg lys pro ile val asp
ACT GCC CAT TTA ATC GAT AGA ACT CGC AAA CCA ATT GTT GAT
315
ala leu lys gln ala lys ile glu ala ser asp leu asp glu
GCT CTA AAA CAA GCA AAA ATT GAG GCT TCA GAT CTT GAT GAA
330
val leu leu val gly gly ser thr arg met pro ala val gln
GTT CTC CTT GTA GGT GGA TCA ACA AGA ATG CCA GCT GTT CAG
345
ser met ile glu his thr leu asn lys lys pro asn arg ser
TCA ATG ATT GAG CAT ACT TTA AAT AAA AAG CCA AAT CGT TCA

FIGURE 5-3

360
ile asn pro asp glu val val ala ile gly ala ala ile gln
ATT AAT CCT GAT GAG GTA GTC GCA ATT GGT GCT GCA ATT CAA
375
gly gly val leu ala gly glu ile ser asp val leu leu leu
GGG GGG GTT CTA GCT GGA GAG ATC AGT GAT GTT CTA CTT TTA
390
asp val thr pro leu thr leu gly ile glu thr leu gly gly
GAT GTT ACT CCT TTA ACT TTA GGA ATT GAA ACT TTA GGT GGA
405
ile ala thr pro leu ile pro arg asn thr thr ile pro val
ATT GCA ACA CCT TTG ATT CCA AGA AAT ACA ACA ATT CCG GTA
420
thr lys ser gln ile phe ser thr ala glu asp asn gln thr
ACA AAA TCA CAA ATT TTC TCA ACA GCT GAG GAT AAT CAA ACC
435
glu val thr ile ser val val gln gly glu arg gln leu ala
GAA GTA ACA ATT TCT GTT GTC CAA GGT GAA CGT CAA CTT GCA
450
ala asp asn lys met leu gly arg phe asn leu ser gly ile
GCG GAT AAT AAA ATG TTA GGT CGC TTT AAT TTA TCA GGA ATT
465
glu ala ala pro arg gly leu pro gln ile glu val ser phe
GAA GCT GCT CCA CGA GGT CTT CCC CAG ATT GAA GTT AGT TTT
480
ser ile asp val asn gly ile thr thr val ser ala lys asp
TCA ATT GAT GTC AAC GGG ATT ACA ACG GTT TCA GCA AAA GAT
495
lys lys thr gly lys glu gln thr ile thr ile lys asn thr
AAA AAA ACC GGC AAA GAA CAA ACA ATT ACA ATT AAA AAT ACT
510
ser thr leu ser glu glu glu ile asn lys met ile gln glu
TCA ACT TTA TCA GAA GAA GAA ATT AAT AAG ATG ATT CAG GAA
525
ala glu glu asn arg glu ala asp ala leu lys lys asp lys
GCC GAA GAA AAT CGT GAA GCT GAT GCT CTT AAA AAA GAC AAA

FIGURE 5-4

```
540
ile glu thr thr val arg ala glu gly leu ile asn gln leu
ATC GAG ACA ACA GTT CGT GCC GAA GGG CTT ATT AAT CAA CTT
555
glu lys ser ile thr asp gln gly glu lys ile asp pro lys
GAG AAA TCA ATA ACT GAT CAA GGT GAA AAA ATT GAT CCA AAA
570
gln lys glu leu leu glu lys gln ile gln glu leu lys asp
CAA AAA GAA TTA CTT GAA AAA CAA ATT CAA GAA TTA AAA GAT
585
leu leu lys glu asp lys thr asp glu leu lys leu lys leu
CTT CTA AAA GAA GAT AAA ACT GAC GAA TTA AAA TTA AAA TTA
600
asp gln ile glu ala ala ala gln ser phe ala gln ala thr
GAC CAA ATT GAA GCA GCT GCC CAA TCT TTT GCG CAG GCA ACC
615
ala gln gln ala asn thr ser glu ser asp pro lys ala asp
GCG CAG CAA GCA AAT ACA TCT GAA TCT GAT CCA AAA GCT GAT
630
asp ser asn thr ile asp ala glu ile lys gln asp OC
GAT TCA AAC ACA ATT GAT GCT GAA ATC AAG CAG GAT TAA
```

FIGURE 11-1

Translation of M. gallisepticum 67 kD Antigen Gene

1
met ser asn asn asn gly leu ile ile gly ile asp leu gly
ATG TCT AAT AAT AAT GGA TTA ATT ATT GGA ATT GAT CTT GGT
15
thr thr asn ser cys val ser val met glu gly ala gln lys
ACC ACC AAC TCT TGT GTG TCT GTA ATG GAA GGT GCA CAA AAA
30
val val ile glu asn pro glu gly lys arg thr thr pro ser
GTA GTA ATT GAA AAC CCA GAA GGT AAA AGA ACT ACT CCA TCA
45
val val ser tyr lys asn gly glu ile ile val gly asp ala
GTA GTT TCA TAC AAA AAC GGT GAA ATT ATT GTT GGT GAT GCT
60
ala lys arg gln met leu thr asn pro asn thr ile val ser
GCT AAG CGT CAA ATG CTA ACT AAC CCA AAC ACT ATT GTT TCT
75
ile lys arg leu met gly thr ser lys lys val lys ile asn
ATT AAG CGT TTA ATG GGA ACA AGT AAA AAA GTT AAG ATT AAT
90
asp lys gly val glu lys glu leu thr pro glu glu val ser
GAC AAA GGT GTA GAA AAA GAA CTT ACT CCA GAA GAA GTT TCT
105
ala ser ile leu ser tyr leu lys asp tyr ala glu lys lys
GCT AGC ATC TTA AGT TAT CTT AAA GAT TAC GCT GAA AAG AAA
120
thr gly gln lys ile ser arg ala val ile thr val pro ala
ACT GGT CAA AAG ATT TCA AGA GCT GTA ATT ACT GTT CCA GCT
135
tyr phe asn asp ala glu arg gln ala thr lys thr ala gly
TAC TTC AAC GAC GCT GAA CGT CAA GCT ACT AAA ACT GCT GGT
150
lys ile ala gly leu thr val glu arg ile ile asn glu pro
AAG ATT GCT GGT TTA ACT GTA GAA AGA ATT ATT AAC GAA CCT

FIGURE 11-2

165
thr ala ala ala leu ala tyr gly ile asp lys gly his arg
ACA GCA GCT GCA TTA GCT TAT GGT ATT GAT AAA GGT CAC CGT
180
glu met lys val leu val tyr asp leu gly gly gly thr phe
GAG ATG AAA GTT CTT GTG TAC GAC CTT GGT GGT GGT ACG TTT
195
asp val ser leu leu asp ile ala asp gly thr phe glu val
GAC GTT TCA TTA CTT GAT ATT GCT GAT GGT ACT TTC GAA GTT
210
met ala thr ala gly asp asn arg leu gly gly asp asp trp
ATG GCT ACT GCT GGT GAT AAC AGA TTA GGT GGT GAT GAC TGA
225
asp asn lys ile ile glu trp ile ile ala glu ile lys lys
GAT AAT AAG ATT ATT GAA TGG ATC ATT GCT GAA ATC AAA AAA
240
asp his pro ser leu asp leu lys ser asp lys met ala met
GAT CAC CCA TCA TTA GAC CTT AAG TCT GAT AAG ATG GCA ATG
255
gln arg leu lys glu ala ala glu arg ala lys ile glu leu
CAA AGA TTA AAA GAA GCT GCT GAA AGA GCT AAG ATC GAA CTA
270
ser ala gln leu glu thr leu ile ser leu pro phe ile ala
TCA GCT CAA TTA GAA ACA CTA ATC TCA TTA CCA TTC ATC GCA
285
val thr pro glu gly pro val asn ala glu leu thr leu ser
GTT ACT CCT GAA GGT CCA GTA AAC GCT GAA TTA ACT TTA TCA
300
arg ala lys phe glu glu leu thr lys asp leu leu glu arg
AGA GCT AAA TTC GAA GAA TTA ACT AAA GAC TTA CTA GAA AGA
315
thr arg asn pro ile ala asp val leu lys glu ala lys val
ACA AGA AAC CCA ATT GCT GAC GTA TTA AAA GAA GCT AAG GTT

FIGURE 11-3

330
asp pro ser gln val asp glu ile leu leu val gly gly ser
GAT CCT AGT CAA GTT GAT GAA ATT CTT TTA GTA GGT GGT TCT
345
thr arg met pro ala val gln lys leu val glu ser met ile
ACA AGA ATG CCT GCA GTA CAA AAA TTA GTT GAA TCA ATG ATT
360
pro asn lys ala pro asn arg thr ile asn pro asp glu val
CCT AAT AAA GCA CCA AAC CGT ACG ATT AAC CCT GAC GAA GTA
375
val ala ile gly ala ala val gln gly gly val leu arg gly
GTA GCG ATC GGT GCT GCT GTA CAA GGT GGG GTA TTA CGT GGG
390
asp val lys asp ile leu leu leu asp val thr pro leu thr
GAT GTT AAA GAC ATC TTA TTA TTA GAC GTA ACT CCT TTA ACA
405
leu ala ile glu thr leu gly gly val ala thr pro ile ile
CTT GCG ATT GAA ACT TTA GGA GGT GTA GCA ACT CCA ATT ATT
420
lys arg asn thr thr ile pro val ser lys ser gln ile phe
AAG AGA AAC ACA ACT ATT CCA GTT TCT AAA TCA CAA ATC TTC
435
ser thr ala gln asp asn gln glu ser val asp val ser ile
TCA ACA GCT CAA GAT AAC CAA GAA TCA GTT GAC GTT TCA ATT
450
tyr gln gly glu arg pro met ala arg glu asn lys ser leu
TAC CAA GGT GAA CGT CCA ATG GCT AGA GAA AAC AAA TCA TTA
465
gly thr phe ser leu gly gly ile gln pro ala pro lys gly
GGA ACT TTC TCA CTT GGA GGA ATT CAA CCA GCT CCT AAG GGT
480
lys pro gln ile glu ile thr phe asn ile asp ala asn gly
AAA CCA CAA ATT GAA ATT ACT TTC AAT ATT GAC GCT AAC GGG

FIGURE 11-4

```
495
ile leu asn val lys ala lys asp leu thr thr gly lys glu
ATT TTA AAT GTT AAG GCT AAA GAC TTA ACA ACT GGT AAA GAA
510
asn ser ile thr ile ser asn ser ser glu leu asp glu asn
AAC AGT ATT ACG ATC TCT AAC TCA AGT GAA TTG GAT GAA AAC
525
glu ile gln arg met ile arg asp ala glu ala asn lys glu
GAA ATC CAA AGA ATG ATC CGT GAT GCT GAA GCT AAC AAA GAA
540
arg asp ala ile val lys gln arg ile glu met arg tyr glu
CGT GAC GCA ATC GTT AAA CAA AGA ATC GAA ATG CGT TAT GAA
555
gly glu gly ile val asn thr ile asn glu ile leu gly ser
GGT GAA GGA ATT GTT AAT ACA ATT AAC GAA ATC CTT GGT TCT
570
lys glu ala glu ala leu pro ala gln glu lys ala ser leu
AAA GAA GCA GAA GCG CTA CCT GCT CAA GAA AAA GCT AGC CTT
585
thr lys ile val asp gly ile asn gly ala leu lys ala glu
ACT AAG ATC GTT GAT GGA ATT AAC GGT GCT CTT AAA GCT GAA
600
lys trp asp glu leu lys glu gln ile asp gly phe lys lys
AAA TGA GAT GAA CTT AAA GAA CAG ATC GAC GGC TTC AAG AAA
615
trp arg asp asp met ser lys lys tyr gly gly gly glu ala
TGA CGT GAT GAC ATG TCT AAG AAA TAC GGT GGT GGC GAA GCT
630
pro ala glu pro lys AM
CCA GCC GAA CCT AAA TAG
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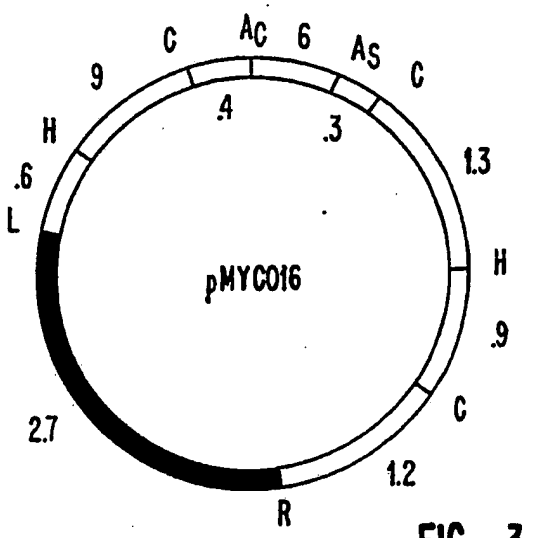
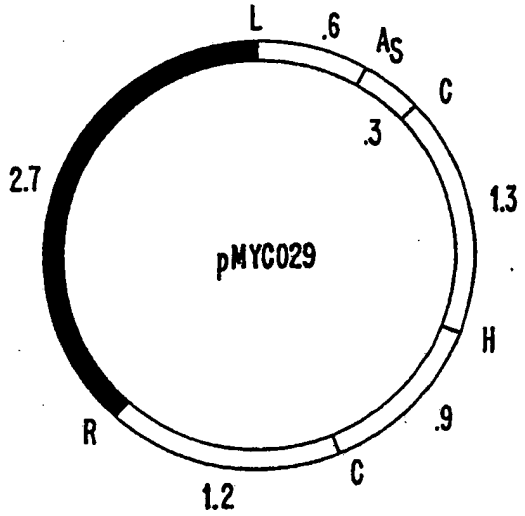


LEGEND

Ac AccI
As AsuII
C ClaI
H HindIII
L HindIII-ClaI-PstI-AccI-EcoRI
R EcoRI

pWHA148
MYCOPLASMA

FIG._3.

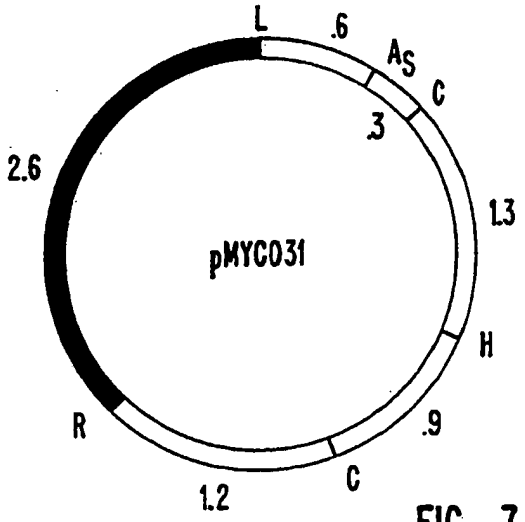


LEGEND

As AsuII
C ClaI
H HindIII
L HindIII-ClaI-PstI
R EcoRI

pWHA148
MYCOPLASMA

FIG._6.



LEGEND

As AsuII
C ClaI
H HindIII
L HindIII-PstI
R EcoRI

pUC9
MYCOPLASMA

FIG._7.

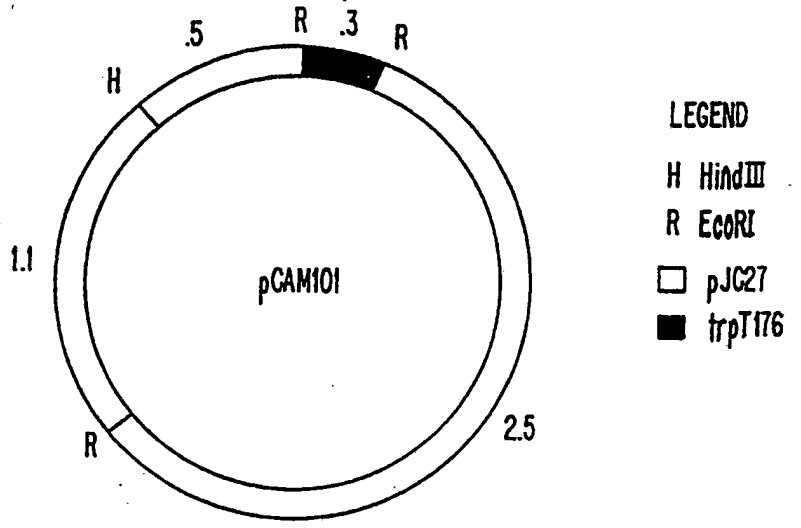

.5
R
.3
R
H
1.1
pCAM101
R
2.5
LEGEND
H HindIII
R EcoRI
pJC27
trpT176

FIG._8.

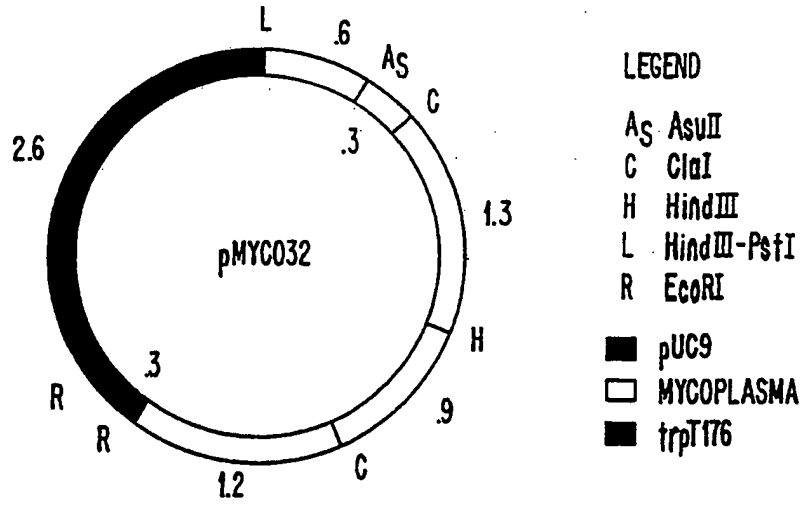

L
.6
AS
C
.3
2.6
1.3
pMYC032
H
.3
R
.9
R
1.2
C
LEGEND
AS AsuII
C ClaI
H HindIII
L HindIII-PstI
R EcoRI
pUC9
MYCOPLASMA
trpT176

FIG._9.

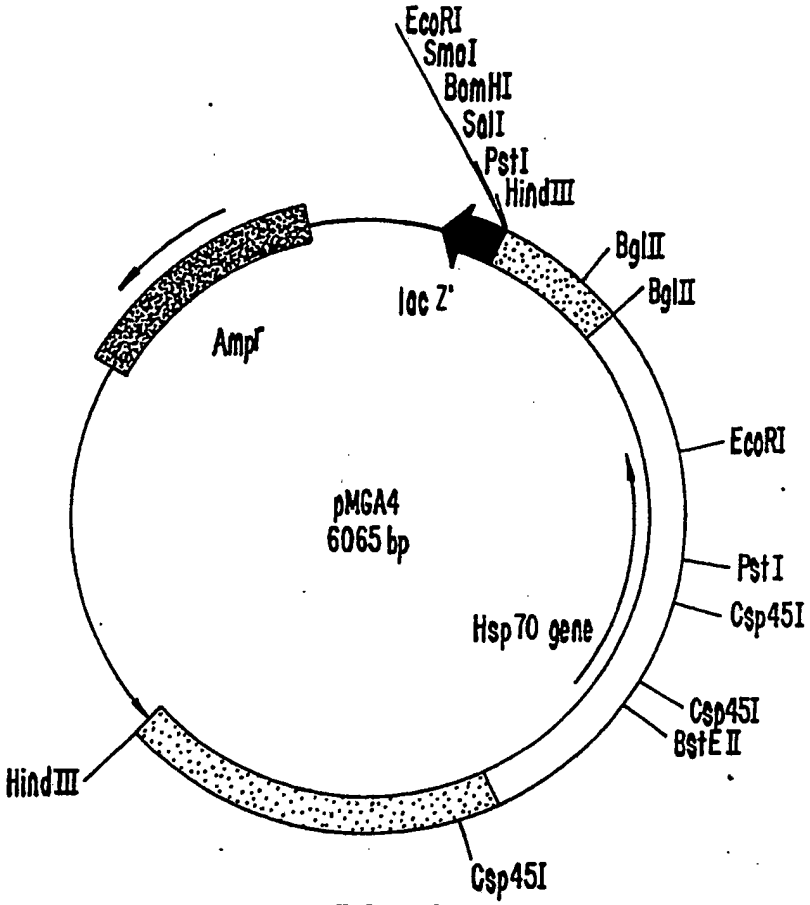

EcoRI
SmaI
BamHI
SalI
PstI
HindIII
BglII
BglII
lac Z'
Amp^r
EcoRI
pMGA4
6065 bp
PstI
Csp45I
Hsp70 gene
Csp45I
BstEII
HindIII
Csp45I

FIG._10.

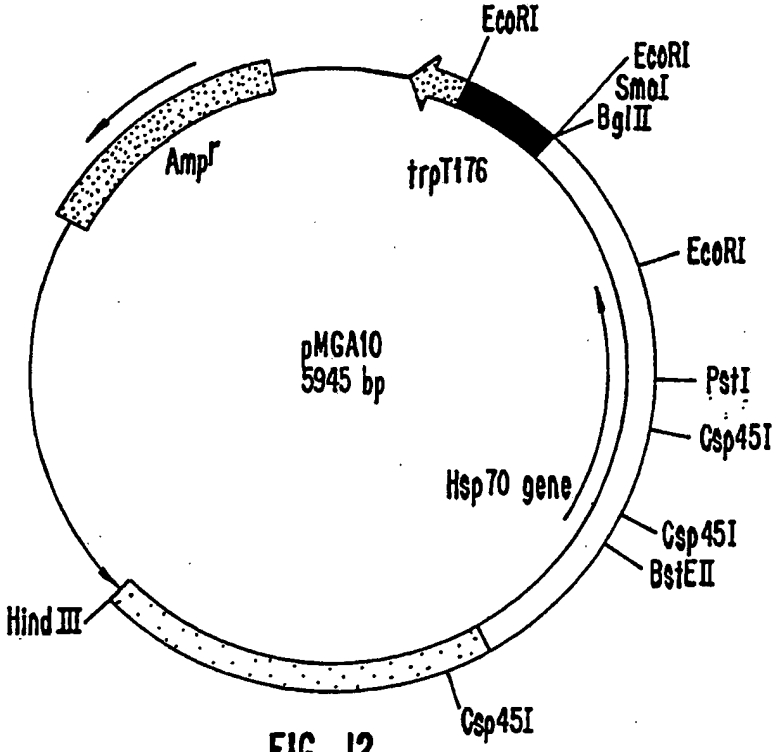

EcoRI
EcoRI
SmaI
BglII
Amp^r
trpT176
EcoRI
pMGA10
5945 bp
PstI
Csp45I
Hsp70 gene
Csp45I
BstEII
HindIII
Csp45I

FIG._12.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03955

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K39/005, 39/04, 39/40; C12N15/00, 1/00; C12P21/00; G01N33/53; A61K39/395

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/88; 435/7, 172.1; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases: Chemical Abstracts Services Online (File CA, 1967-1989; File Biosis, 1969-1989). Automated Patent System (USPAT), 1975-1989). Sequence search (protein databases:PIR,Swiss-Prot).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Molecular and Cellular Biology, Volume 6, Number 12, December 1986, Glass, "Conserved sequences and transcription of the hsp70 gene family in Trypanosoma brucei", pp. 4657-4666.	1-11, 17 12-16
X Y	Cell, Volulme 2, October 1980, Ingolia, "Sequence of three copies of the gene for the major drosophila heat shock induced protein and their flanking regions", pp. 669-679.	1-11, 17 12-16
X Y	Nucleic Acids Research, Volume 15, Number 13, 1987, Dworniczak, "Structure and expression of a human gene coding for a 71 kd heat shock 'cognate' protein", pp. 5181-5197.	1-11, 17 12-16
Y	US, A, 3,993,743 (Hanson) 23 November 1976	12-16
Y	Phil. Trans. R. Soc. Lond., Volume B 307, 1984, Scott, "The vaccine potential of cell	1-17

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 December 1989	10 JAN 1990
International Searching Authority	Signature of Authorized Officer
ISA/US	D. Bernstein

Form PCT/ISA/210 (second sheet) (Rev. 11-87)